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To: STIC-ILL

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I need the following references for 09/831623.

Thanks, Ulrike

Ulrike Winkler, Ph.D.  
Patent Examiner, Art Unit 1648  
Crystal Mall 1, 8D09/ Mail Box 8E12  
1911 South Clark Place  
Arlington, VA 22202  
tel. 703-308-8294  
fax. 703-308-4426

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## Retroviral Vectors for Liver-directed Gene Therapy

GANJAM V. KALPANA, Ph.D.

**ABSTRACT:** Retroviruses are popular gene therapy vectors because they stably integrate the DNA copy of their genome into the host chromosome during their replication cycle. The widely used murine retroviral vector systems have two components: the transfer vector for the transgene carries all the cis-acting elements necessary for the replication and efficient integration of the viral DNA; and the packaging cell line produces all the trans-acting proteins necessary for both structural and catalytic functions of the virus. Advances in design of retroviral vectors have resulted in greater degree of biosafety, expanded host range, and increased stability of the virus particles. Retroviral vectors have been widely used in the *ex vivo* gene therapy protocols to correct the liver diseases in a wide variety of species. In a limited number of applications, *in vivo* gene therapy has been achieved after the liver cells have been stimulated to regenerate. One major limitation of murine retroviral vectors is their inability to infect nondividing cells. This problem has been overcome by deriving vectors from lentiviruses (a class of retroviruses) that have the ability to infect both dividing and nondividing cells. The lentiviral vectors are derived from human immunodeficiency virus type 1 (HIV-1). Initial studies using lentiviral vectors for gene delivery to the liver *in vivo* show promising results. A highly crippled version of lentivirus has been generated by using producer cells in which the trans-acting components are expressed by several different coding elements and vectors that incorporate features of self-inactivation. These improvements should ensure biosafety of lentiviruses and make them useful in efficient delivery of therapeutic genes to nondividing differentiated tissues such as the liver.

**KEY WORDS:** retroviral vectors, gene therapy, lentiviral vectors

Development of methods to deliver therapeutic genes to correct inborn or acquired deficiencies is a key aspect of gene therapy.<sup>1</sup> Of the several modes of gene delivery available today, use of recombinant viruses is the most powerful because it exploits the efficient machinery evolved by viruses to deliver nucleic acids into host cells.<sup>2</sup> Retroviruses, the best studied vectors for gene therapy, not only can deliver the nucleic acid genome, but also stably insert a complementary DNA copy of their genomic RNA into the host chromosome as an essential step of their life cycle.<sup>1</sup> This unique ability to efficiently integrate into host genome and to be faithfully transmitted to the progeny cells has made them the most popular gene therapy vectors. In addition to efficient entry and integration into cells, their broad tissue and host range, expression of transduced genes at high levels, ease of production at reasonably high titers, availability of producer cell lines (the packaging cell

lines), and lack of adverse host immune response to retroviral vectors all add to their inherent advantage for use as vehicles for gene transfer.<sup>3</sup> Recent advances have resulted in lentiviral vectors, which have an unusual property that makes them suitable for noninvasive *in vivo* gene therapy of terminally differentiated tissues such as liver. This property is the ability of lentiviral vectors to infect and integrate into the genome of nondividing cells.<sup>3</sup> In this article, the principles underlying design, construction, and use of retroviral vectors for liver gene therapy will be discussed with a special emphasis on the lentiviral vectors.

### RETROVIRAL VECTORS

#### Salient Features of Retroviral Replication

A thorough understanding of retroviral replication has helped the design of efficient retroviral vectors. Therefore, a brief description of retroviral life cycle will be presented prior to the description of design of retroviral vectors.<sup>4</sup>

From the Department of Molecular Genetics, Albert Einstein College of Medicine, Bronx, New York

Reprint requests: Dr. G. V. Kalpana, Department of Molecular Genetics, Albert Einstein College of Medicine, U821, Bronx, New York, NY-10461. E-mail: kalpana@aecom.yu.edu.

### The Structure of Viral RNA

Retroviruses are RNA viruses. The virus particle contains two identical copies of genomic RNA along with viral and cellular proteins (Fig. 1). There are three major open reading frames—*gag*, *pol*, and *env*—in the viral RNA that encode the structural and catalytic proteins required for various steps of viral replication. The viral RNA is flanked on either side by elements called long terminal repeats (LTRs). The 5' end is flanked by regions R (for repeat) and U5, whereas the 3' ends are flanked by regions U3 and R. The LTRs harbor the *cis*-acting promoter, upstream enhancers as well as polyadenylation signals essential for viral transcription. Additional *cis*-acting elements include signals (acceptor and donor sites) for proper splicing of mRNAs, the packaging sequence ( $\psi$ ) that allows the viral RNA to be specifically packaged into the virus particle and primer-binding sites for the initiation of reverse transcription in the recipient cell (Fig. 1).

### Early Events in Retroviral Replication

Viral entry into the host cell is mediated by a specific interaction of the envelope glycoproteins on the surface of virus particles with receptors on the host cell surface. The receptor-envelope interaction primarily determines host range. Murine amphotropic retroviruses have a broad host range, capable of infecting many different cell types from various species, including mouse and human, and have been the mainstay in the design of gene therapy vectors for humans. After entry into the host cell, the RNA genome is reverse-transcribed in the cytoplasm into double-stranded DNA. During the process of reverse-transcription, the U5 region is transferred to 3' LTR and the U3 region is transferred to 5' LTR in such a way that both LTRs possess U3, R, and U5 regions (Fig. 1).

The double-stranded DNA resulting from reverse transcription resides within a high molecular weight nucleoprotein complex called the preintegration complex (PIC), which includes the machinery necessary for integration of viral DNA. The transport of this large nucleoprotein complex from the cytoplasm into the nucleus of the infected cell is a prerequisite for integration. Unlike lentiviruses, the PICs of murine retroviruses (e.g., Moloney-murine leukemia virus [Mo-MuLV]) are incapable of crossing the nuclear membrane.<sup>5</sup> To access the host genome, they have to await the dissolution of nuclear envelope during mitosis. Thus, integration of murine retroviruses is dependent on cell division. Because integration is essential for their replication, murine retroviral vectors cannot infect nondividing cells and terminally differentiated tissues.

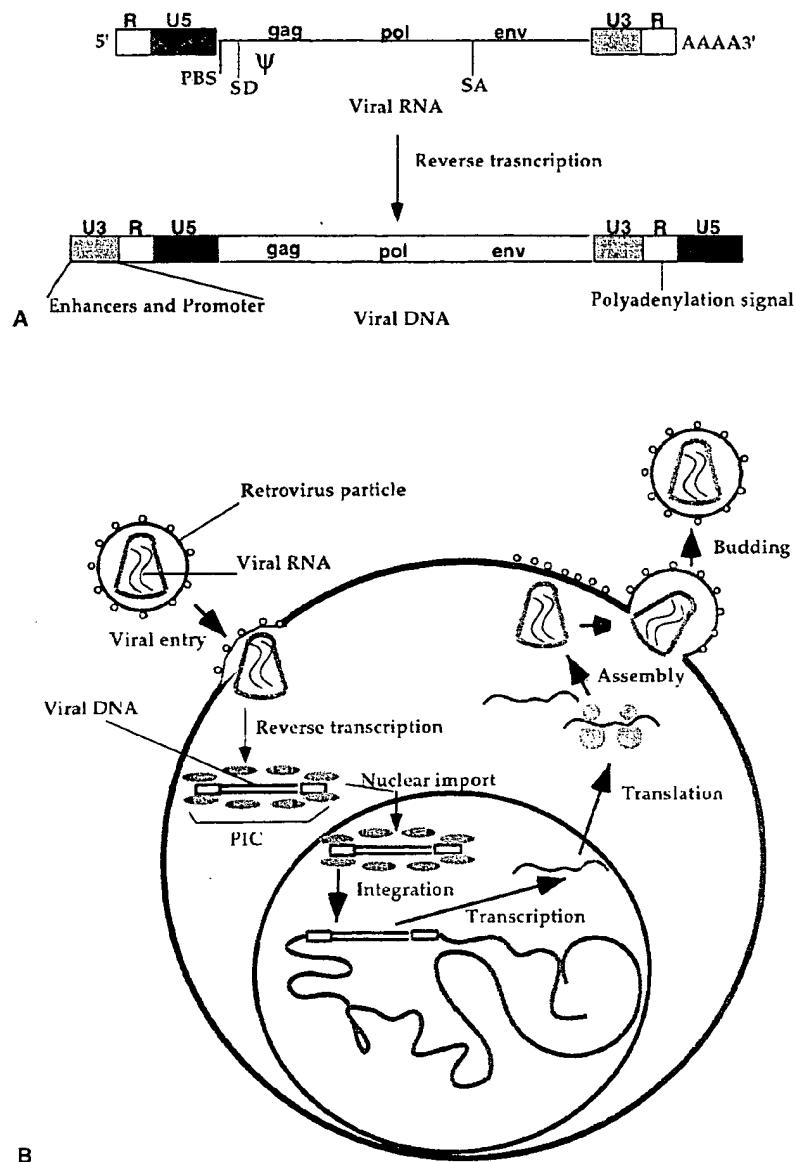
### Replication-defective Retroviral Vectors

The most effective retroviral vectors are those that are capable of incorporating therapeutic genes, delivering them to the host cells via a single cycle of infection, and incapable of further spread. Such replication-defective viral vectors are derived by manipulating the viral genome and by replacing most or all of the viral genes by therapeutic transgenes.<sup>1</sup> The transgenes are replicated and integrated into the host genome along with the essential *cis*-acting elements of retroviral vectors. Generation of such virus particles with a mixed composition of partially defective genomic RNA encapsidated within functional viral proteins is possible because viral proteins necessary for processes associated with entry, reverse-transcription, and integration can be provided *in trans* by the helper cell lines, called the packaging cell lines. Because there is no need for viral protein synthesis during the early events of infection, the replication-defective virus produced in the packaging cell line can infect the recipient cell and integrate the vector DNA into the host genome without the *de novo* synthesis of viral proteins. Once integrated, it is no longer able to propagate in the absence of virus-specific genes. These replication-defective vectors are often called transducing viruses to distinguish them from replication-competent viruses.<sup>1</sup>

The replication-defective, transducing viruses are produced in two stages. First, the therapeutic gene of interest is cloned into the transducing vector DNA that is "crippled" or deleted of all the viral genes necessary for replication, such as *gag*, *pol*, and *env*, while retaining the " $\psi$ " signals and other *cis* sequences necessary for the packaging of recombinant RNA molecule into the virus particle, and subsequent reverse-transcription and replication. Second, the recombinant viral DNA is transiently transfected into a packaging cell line that expresses the viral *gag*, *pol*, and *env* proteins *in trans* via a second defective viral genome, called the helper virus, which is devoid of *cis*-acting sequences, including  $\psi$  sequences (Fig. 2).

### Generating Retroviral Vectors with a High Degree of Biosafety

Early helper vectors contained simple deletions of the  $\psi$  region and were capable of producing all viral proteins, including Gag, Pol, and Env. Although the helper virus genome itself cannot be packaged, the recombination of helper virus with the transducing vectors leads to a possibility for regenerating a replication-competent recombinant (RCR).<sup>6</sup> This has led to the design of multiply attenuated helper viruses. In some of the systems, the 3'-LTR of the helper virus, whose sole function was to provide polyadenylation signal, was re-

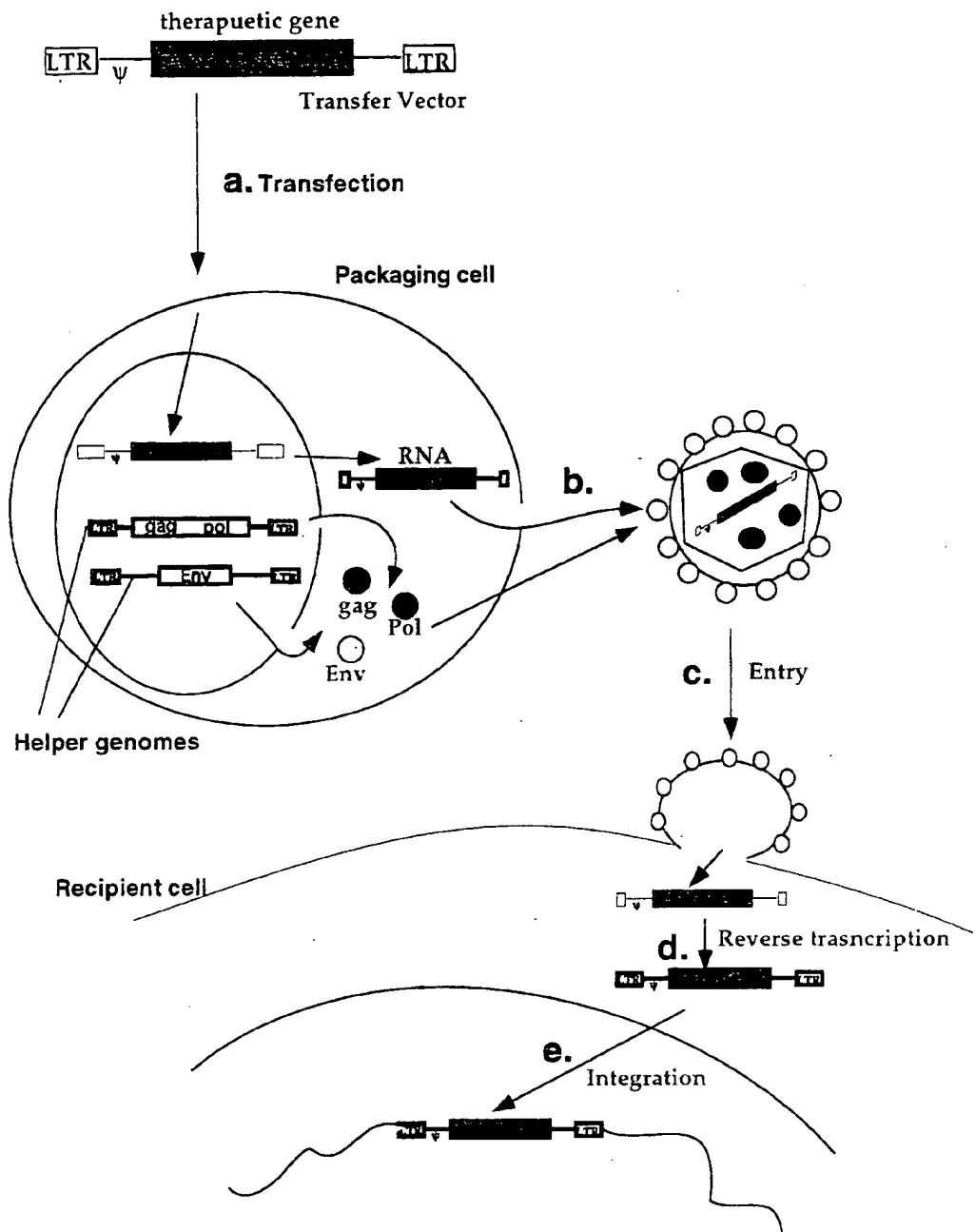


**FIG. 1. Structure of genome and replication cycle of retroviruses.** (A) Retroviral genomic RNA comprises three open reading frames for genes *gag*, *pol*, and *env* and is flanked at each end by LTRs. The 5' LTR consists of regions R and U5; the 3' LTR comprises regions U3 and R. Upon reverse transcription a double-standard (ds) DNA copy of the virus is formed that carries all the regions of viral RNA. In addition, each LTR end consists of three regions: U3, R and U5. Whereas the 5' U3 region provides enhancers and promoter elements for the transcription of the integrated copy of the virus, the R region provides polyadenylation signals.  $\psi$  = packaging signal; PBS = primer binding site; SD = splice donor; SA = splice acceptor. (B) Retroviral life cycle. Viral RNA that is encapsidated in the particles enters the cytoplasm, where reverse transcription converts it into ds DNA. This DNA resides within a high molecular weight nucleo-protein complex called the preintegration complex (PIC). Upon entry of the PIC to the nucleus, integration of viral DNA to host DNA takes place. Subsequent steps of transcription of integrated viral DNA, translation, assembly, and budding releases the progeny virus particle.

placed with non retroviral polyadenylation signals, thus reducing the probability of recombination. A higher level of safety also has been ensured by using split genomes, where *gag/pol* and *env* are separated on two distinct helper genomes. This further reduces the proba-

bility of generating RCRs by increasing the number of crossovers required for recombination.

When split genomes are used, it is possible to pseudotype the transducing viruses by mixing the *gag/pol*-encoding genome from one virus with an *env*-



**FIG. 2. Schematic representation of retroviral gene therapy vector system.** (A) The gene of interest (therapeutic gene) is first cloned into the transfer vector carrying the *cis*-acting elements and the recombinant DNA is transfected into producer cells known as packaging cells. (B) Packaging cells carry helper genomes but, lacking the  $\psi$  signal, express the viral proteins that assemble along with the RNA from the transfer vector to produce viral particles. (C) The virus particles carrying vector RNA enters the cells. (D) The transfer vector along with the transgene is converted into DNA by reverse transcription. (E) Integration of the vector DNA results in the stable insertion of therapeutic genes in the recipient cells.

encoding genome from a different virus with different host range.<sup>1</sup> Pseudotyped viruses acquire the host range properties of the virus from which the envelope is derived but displays the replication capability of the virus from which the remaining sequences are derived. In general, the transducing viruses are pseudotyped with envelope from amphotropic retroviruses.

### Self-inactivating Retroviral Vectors

In combination with the multiply attenuated helper vectors with split genome, an additional biosafety feature has been introduced by making the transfer vector handicapped or self-inactivating.<sup>7-9</sup> This is possible because of the modality of retroviral reverse-transcription, where the U3 region of both 5' and 3' LTRs of the viral DNA are derived from the U3 region of the 3'LTR of the viral RNA (Fig. 3). The U3 region carries promoters and enhancer elements necessary for the transcription of integrated provirus. A deletion introduced into the U3 region to remove enhancer and promoter elements at the 3' end of the transfer vector gets transmitted to the 5' region of the viral DNA in the recipient cell during reverse-transcription. When this viral DNA integrates, it lacks both 5' and 3' U3 regions, and thus is not able to initiate transcription from the viral LTRs (Fig. 3). When using self-inactivating (SIN) retroviral vectors, the transgene is always expressed from an internal promoter. This design of SIN vectors also allows the use of cognate promoters as internal promoters because there will not be transcriptional interference from the 5' LTRs. The second advantage of using SIN vectors is that the integrated proviral genome is not capable of activation of an adjacent cellular oncogene due to the lack of 3' promoters, thus increasing the biosafety measures.

An extensive list of all the available packaging cell lines carrying different helper viruses can be found elsewhere.<sup>1</sup>

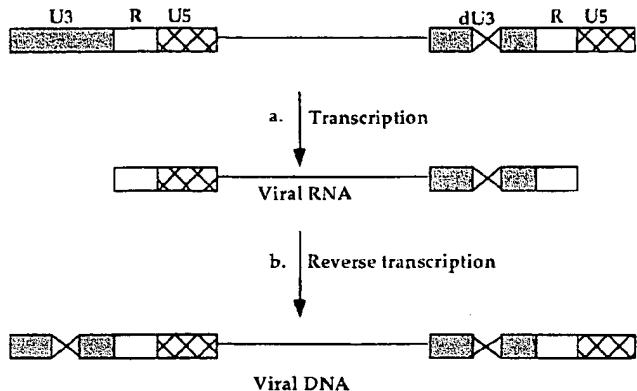
### Gene Therapy of Liver Using Retroviral Vectors

Standard murine retroviral vectors are well suited for ex vivo gene therapy applications of nondividing cells such as those in liver tissue. Hepatocytes from a variety of species, including mice, rats, rabbits, baboons, and humans, have been transduced with therapeutic genes using retroviral vectors ex vivo.<sup>10-14</sup> The integrating characteristic of retroviruses was exploited in the first successful long-term ex vivo gene therapy for a metabolic disease of the liver. In this study, hepatocytes were isolated from a resected liver lobe of low-density lipoprotein (LDL) receptor-deficient Watanabe heritable hyperlipidemic (WHHL) rabbits. The primary

hepatocytes were established in culture and transduced with a MoMuLV-based vector expressing human LDLR.<sup>10</sup> Phenotypic correction of the transduced cells was demonstrated by reconstitution of the ability to internalize LDLs. Following retrovirus-mediated transduction, the cells were transplanted back into the donor rabbit by infusion into the portal vein.<sup>10</sup> Because the hepatocytes were autologous, no immunosuppression was needed. Long-term survival and function of the transduced transplanted cells was demonstrated by molecular analysis of liver biopsy specimens. The transplantation resulted in a 20 to 35% reduction of serum LDL cholesterol levels for the duration of the study (6 months).<sup>10</sup> Following this preclinical study, this ex vivo method was evaluated in a clinical study in four patients with familial hypercholesterolemia.<sup>15,16</sup> Although the reduction of serum LDL cholesterol was modest, and probably not therapeutically adequate, these studies showed the feasibility of transplanting transduced autologous liver cells in a clinical setting.<sup>17</sup>

Efficiency of ex vivo gene therapy using autologous primary hepatocytes is restricted by several factors. First, the number of hepatocytes that can be harvested from liver segments resected from a patient is limited. Second, the transduction of cultured primary hepatocytes, which divide only infrequently, is inefficient. Third, only a limited number of hepatocytes, representing 1 to 5% of the total hepatocyte mass, can be transplanted into the liver at one time.<sup>18,19</sup> In cases where the phenotypically normal transplanted cells have a definite survival advantage over the host hepatocytes, it is expected that the host liver eventually will be repopulated by a small number of transplanted hepatocytes. For example, in hereditary tyrosinemia type 1 (fumarylacetoacetate hydrolase deficiency) and progressive familial intrahepatic cholestasis type 3 (MDR3 abnormality) the life span of the host liver cells are markedly reduced. This should provide a selective proliferative pressure on the phenotypically normal transplanted cells, which should eventually replace the abnormal cells. These aspects are discussed more fully in the article by Grompe et al. in this issue.

To overcome some of the hurdles described above, two-stage retroviral transduction of hepatocytes has been performed in rats. Hepatocytes were harvested from a resected liver lobe of a bilirubin-UDP-glucuronosyltransferase-deficient (bilirubin-UGT) jaundiced Gunn rat.<sup>20</sup> As a first step, primary hepatocytes were transduced with a retroviral vector expressing a thermostable mutant simian virus large T-antigen. This results in conditional immortalization of the hepatocytes. The cells proliferate in culture at 33°C. At physiologic temperatures, the mutant T-antigen is degraded, and the cells stop proliferating and express characteristics of differentiated hepatocytes.<sup>21</sup> During the proliferative phase of the cells, they were efficiently transduced using a second



**FIG. 3. Self-inactivating (SIN) vectors.** These vectors carry a deletion of the essential enhancer and promoter elements in the U3 region (dU3) at the 3' end of the LTR of the transfer vector. (A) Transcription of the vector in the producer cell line results in viral RNA that only carries the deleted U3 region but not the 5' U3 region. (B) Reverse transcription of this RNA in the recipient cells transfers the deletions at the 3' U3 region to the 5' region of the resulting viral DNA. This deleted U3 region is not able to transcribe viral DNA, and hence is self-inactivating.

recombinant retrovirus, expressing human bilirubin-UGT.<sup>20</sup> The phenotypically corrected transduced cells were cloned, expanded in culture, and transplanted into syngeneic Gunn rats in five different sessions. This resulted in approximately 30% reduction in serum bilirubin levels on a long-term basis. The transplanted immortalized hepatocytes were not tumorigenic, either in the Gunn rats or in immunodeficient (SCID) mice.

In some animal experiments, *in vivo* gene transfer to the liver has been accomplished using retroviruses.<sup>21</sup> Hepatocellular proliferation was induced in Gunn rats by 66% hepatectomy 24 hours before delivery of retroviral vectors. The inflow and outflow vessels of the liver were clamped and catheterized. The livers were perfused in situ with a recombinant retrovirus expressing human bilirubin-UGT. This resulted in sustained reduction of serum bilirubin levels for 18 months (duration of the study). Replacement of partial hepatectomy by other noninvasive approaches for the induction of hepatocellular proliferation is being investigated. These strategies include the administration of tri-iodothyronine, hepatocyte growth factor, lipopolysaccharides, and activators of Fas.<sup>22-25</sup>

### PROGRESS IN DESIGN OF RETROVIRAL VECTOR SYSTEM

There are several limitations to the commonly used vector/packaging systems for *in vivo* gene therapy of liver and other nondifferentiated tissues. First, it is difficult to concentrate these viruses to achieve high titers as they are disrupted or ruptured during high-speed centrifugation. Second, amphotropic host range virus is inactivated by human serum. Third, they are unable to integrate in nondividing cells. Fourth, there is a possibility of transcriptional shut-off that occurs *in vivo* after prolonged periods. Recent advances in gene therapy strategies have resulted in overcoming many of these problems, which are discussed in the following sections.

### Production of Highly Concentrated Retroviral Vector Particles

This has been achieved by pseudotyping the virus particles with envelope G glycoprotein of vesicular stomatitis virus (VSV), a class of RNA virus.<sup>26,27</sup> VSV-G protein was coexpressed in place of murine envelope protein, along with the helper virus that is devoid of *env* gene and transducing vector in the packaging cells. The resulting pseudotyped virus particles that had incorporated the VSV-G envelope possessed a wide host range of VSV and could be highly concentrated by centrifugation without loss of biological activity. Titers as high as 10<sup>8</sup> per ml could be achieved using this method.

### Packaging Cell Lines with Inducible Synthesis of VSV-G

Initial studies used transient transfection to express VSV-G protein in the packaging cells because the constitutive expression of high levels of VSV-G in most cells is toxic. This significantly limits the application of pseudotyped viruses because only small amounts of the viruses could be produced at a given time. This toxicity problem has been overcome by developing human-derived packaging cell lines that express VSV-G under a tetracycline-inducible promoter.<sup>28</sup> In this case, the packaging cells harbor an altered gene encoding VSV-G protein under the *tet*<sup>0</sup> promoter, which is repressed by *tet*/VP16 protein in the presence of tetracycline. The *tet*/VP16 repressor is expressed from a second stably integrated construct and is inactivated upon the removal of tetracycline from the medium. In addition to these two constructs, the Gag-Pol proteins were expressed from a cytomegalovirus (CMV) promoter in the third construct. To generate retroviral vectors, the transducing vectors are first stably introduced into these packaging cells and cultured to obtain a desired cell density. Once the desired density of cells is

obtained, tetracycline-containing medium is removed and replaced by medium without tetracycline to inactivate the repressor. This results in the production of a large amount of VSV-G pseudotyped virus particles in the culture supernatants that can be concentrated 1000-fold, resulting in viral titers up to  $10^9$  infectious particles per ml.

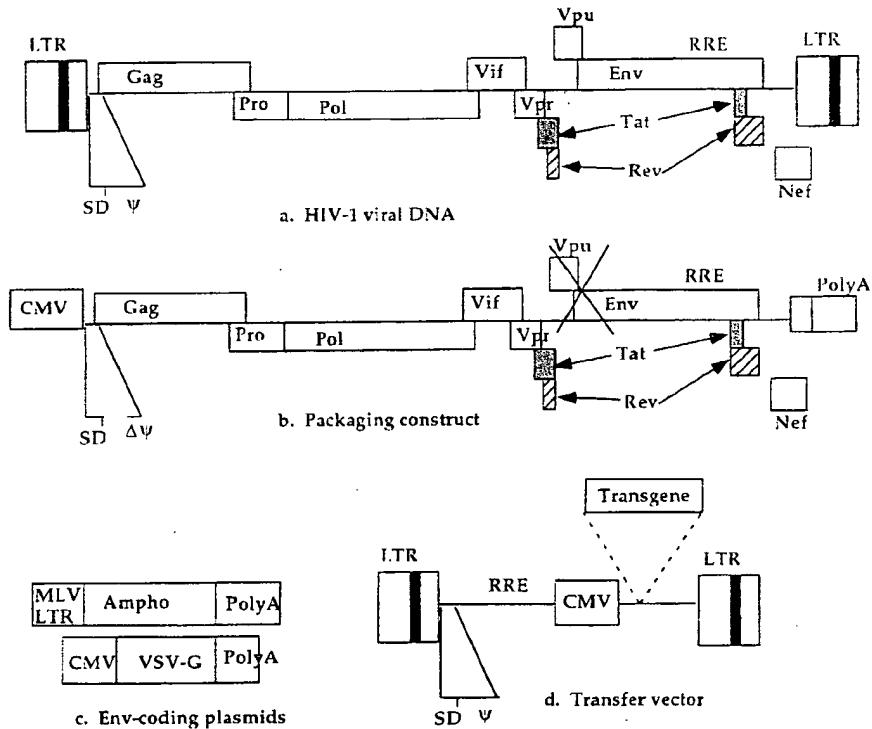
Use of human-derived cells as packaging cell lines and incorporation of VSV-G proteins into retroviral vector has additional advantages. Unlike amphotropic envelopes, the VSV-G pseudotyped retroviral vectors were shown to be significantly more resistant to human serum.

### LENTIVIRAL VECTORS

Unlike murine retroviruses, lentiviruses, a class of complex retroviruses, are capable of infecting both actively dividing as well as nondividing cells.<sup>29</sup> This property makes them most desirable for use in noninvasive in vivo gene therapy. Although the basic mode of replication of lentiviruses is similar to that of murine retroviruses, they differ in many aspects; hence, the life cycle of lentiviruses is discussed here, highlighting the differences followed by a description of latest advances in developing lentiviral vectors.

### Salient Features of the Lentiviral Life Cycle

The best studied lentivirus is HIV-1. A crippled version of this virus has been developed into a lentiviral vector and has been used as a vehicle for in vivo gene delivery.<sup>30</sup> Like simple retroviruses, the genomic RNA of HIV-1 contains three major genes: *gag*, *pol*, and *env*. In addition to these, it also carries open reading frames for six accessory genes: *tat*, *rev*, *vpr*, *vpu*, *nef* and *vif*.<sup>31,32</sup> These accessory proteins are encoded by multiple exons in the viral genome and thus require multiple splicing of viral RNA for production (Fig. 4). The Tat and Rev proteins are important for the efficient gene expression at the transcriptional and posttranscriptional levels, respectively. Tat dramatically increases HIV-1 transcription by binding to TAR (transactivation response) sequences in the nascent RNA. TAR is a stem loop structure upon binding, to which Tat protein stimulates transcriptional elongation by RNA *Pol II*. Rev protein is essential for the export of unspliced and singly spliced viral transcripts. After transcription, the Rev protein binds to sequences in the viral RNA known as RRE (Rev response elements) and facilitates the nuclear export and cytoplasmic accumulation of viral transcripts.



**FIG. 4. Structure of HIV-1 viral DNA and the three plasmid based lentiviral vector system. (A)** Structure of HIV-1 DNA indicating the open reading frames for major proteins and accessory proteins. **(B)** Packaging construct of the helper virus provides all the viral proteins except Env; there is a stop codon at the beginning of Env and a deletion in the packaging signal ( $\Delta\Psi$ ). **(C)** Envelope proteins, Amphi or VSV-G, are expressed from a separate plasmid vector. **(D)** The transfer vector carries all the *cis*-acting elements. It also carries an internal CMV promoter for the expression of transgene.

Both Tat and Rev are indispensable for the production of progeny virus particles. However, the remaining four accessory proteins Vif, Vpu, Vpr, and Nef are not essential for viral replication. Some of these, such as Vif and Nef, are required for *in vitro* viral replication in a cell type-specific manner. Some of them, such as Vpr and Nef, are thought to be necessary for pathogenesis *in vivo*. The mechanism of action of the above four accessory proteins are still not completely understood.<sup>31,32</sup>

The most striking feature of HIV-1 is its ability to replicate in nondividing cells. This property is due to the ability of the lentiviral PICs to get actively transported to the nucleus when the nuclear membrane is still intact.<sup>29</sup> The nuclear import of PICs is an ATP-dependent process and is mediated by the mutually exclusive nuclear localization signals in *vpr* and matrix protein (MA, which is encoded by *gag*). In addition, the integrase (IN protein, encoded by *pol*) is also thought to play an important role in nuclear localization. Thus, lentiviruses can get access to cellular DNA during all stages of the cell cycle, and integration of the viral DNA can proceed in the absence of active cell division.

### Generating Lentiviral Vectors

Using retroviral vectors as models, lentiviral vectors have been generated by inserting the transgene between the LTRs and the packaging signal. The env protein of HIV-1 would limit the target cell specificity to T cells and macrophages. Therefore, similar to murine-retroviral vectors, the lentiviral vectors are pseudotyped with VSV-G to obtain broad host range. The VSV-G envelope significantly increases the stability of virus particles and hence allows the concentration of the lentiviral preparations by centrifugation as well. The use of VSV-G envelope does not induce cellular immune response or inflammations *in vivo*.

One of the first-generation lentiviral vectors developed is a three-plasmid-based system that includes a helper construct, an envelope-encoding construct, and the transducing vector (Fig. 4).<sup>30</sup> In this system, the helper construct lacks the viral 3' and 5' LTRs,  $\psi$  sequences, envelope-coding regions, and expresses the *gag*, *pol*, and accessory proteins from a CMV promoter. The *env*-coding plasmid expresses either an amphotropic envelope or a VSV-G envelope for pseudotyping. The transducing vector or transfer vector contains *cis*-acting sequences of HIV-1 necessary for reverse transcription and integration (3' and 5' LTRs and primer binding site), packaging sequences, splice signals, RRE for nuclear export, and unique restriction sites for cloning the transgene of interest. In addition to these *cis*-acting sequences, an internal CMV promoter and ribosomal entry site (RES) have been incorporated to facilitate the transcription of transgene in the absence of viral accessory proteins in the recipient cell (Fig. 4).

To generate the replication-defective virus particles, the three plasmids are transiently cotransfected into human 293T cells. All viral proteins are produced by the helper and envelope constructs, and the transducing RNA is produced by the transfer vectors, which are then assembled into virus particles and released into the supernatant. Virus particles in the culture supernatants can be concentrated by centrifugation if pseudotyped with VSV-G. These viral supernatants are then applied to desired tissues for gene delivery. As in retroviral vectors, proteins packaged in the virus particles are sufficient for the entry, reverse-transcription, and integration of transfer vector in the recipient cell. In the absence of viral proteins, the transfer vector is defective for viral replication, and only the transgene is expressed from an internal promoter.

### Using Lentiviral Vectors for Liver Gene Therapy

The efficacy of the first-generation lentiviral vectors for *in vivo* liver gene therapy has been tested.<sup>33</sup> In this case, an *eGFP* (eukaryotic humanized green fluorescent protein) gene was cloned into the transfer vector upstream of a CMV promoter. Virus particles prepared as described above and pseudotyped with VSV-G protein were injected into the liver parenchyma of adult female nude rats. The transduction efficiency was determined by monitoring green fluorescence. About 3 to 4% of total liver tissue was transduced by a single injection of 1 to  $3 \times 10^7$  infectious units of recombinant lentiviral vector. GFP was detected for a period of 6 months, the longest period tested after injection. No detectable inflammation was observed at the site of injection in the liver, indicating that there is no host mediated cellular immune response to lentiviral vectors. On the contrary, the MuLV-based vector used as a control did not show any transduction of liver cells *in vivo*. These results clearly indicate the usefulness of lentiviral vectors for *in vivo* liver gene therapy.

### Progress in Design of Lentiviral Vectors

A major limitation in using lentiviral vectors for gene therapy is the biosafety consideration of introducing pathogen-derived sequences into humans. The concern is that the segregated *cis* elements of transfer vector could recombine with the *trans*-acting elements of the helper construct, or even more likely, with the endogenous retroviral sequences to regenerate RCR and present a risk to the recipient. The split-genome design of the three-plasmid vector described already ensures that such events are highly improbable because multiple crossover events need to take place in the correct order to regenerate RCR. Additionally, because the endoge-

nous retrovirus sequences present in the host genome do not have any nucleotide sequence homology to the lentiviral class of retroviruses, the probability of their recombination to generate RCR is minimal. Furthermore, one can also consider using simian or other lentiviruses as vectors, which will further reduce the possibility of homologous recombination. Improvements in the vector design have resulted in multiply attenuated vectors and self-inactivating vectors that should provide important additional safety features.

### Multiply Attenuated Lentiviral Vectors

The second- and third-generation lentiviral vectors were generated by removing many of the accessory proteins from the vector system.<sup>34</sup> As discussed earlier, these accessory proteins are encoded by the helper construct. By introducing mutations or deletions, four of the accessory proteins that are not essential for vector-mediated transduction—Vpr, Vpu, Vif and Nef—were eliminated. It is known that the function of Tat is necessary in the packaging cells only for LTR-mediated transcription of transfer vector. However, if a part of the promoter present in the U3 region of LTR was replaced by a constitutive promoter such as CMV, the Tat dependency could be completely removed. Thus, five of the six accessory proteins can be eliminated. Moreover, the helper virus coding region was further split by removing the Rev coding region and providing it on a fourth plasmid.<sup>35</sup> Such highly crippled vectors both provide all the necessary *trans*-acting functions and are much safer. However, while using these vectors it is important to determine the requirement for the specific tissue in question. For example, it was noticed that efficient transduction in liver requires Vif and Vpu proteins, but they are not required for transduction in neuronal cells.<sup>33</sup> Although the mechanism of these differences is not known, it is thought that tissue-specific host cell proteins may complement the absence of these proteins in some cell types but not others.

### Self-inactivating Vectors

Recently, SIN lentiviral vectors have been developed where most of the U3 (a 400-base pair) region of LTR, including the entire promoter region, has been removed.<sup>36,37</sup> In one case, only 53 nucleotides were left for (1) proper recognition and processing by integrase encoded by *pol* and (2) polyadenylation. This vector has all the functions necessary for transduction but is self-inactivated because deletion in the U3 region at the 3' end of the viral RNA is transmitted to the 5'LTR end of the viral DNA during reverse-transcription. Thus, the integrated viruses are nonfunc-

tional due to extensive deletions in both the 3' and 5' U3 regions (Fig. 3).

## PERSPECTIVES

Retroviral vectors offer great potential for gene therapy. Of several hundred clinical gene therapy trials currently ongoing, more than 45% are using retroviral vectors. Only a handful of clinical trials are directed to correcting liver diseases, including familial hypercholesterolemia, liver metastasis, and acute liver failure (Wiley Clinical Trials Database). The major limitation in using murine-based retroviral vectors for liver-directed gene therapy, as discussed earlier, is their inability to infect nondividing cells. This problem can be solved by using lentiviral vectors because they can infect nondividing terminally differentiated tissues such as liver while retaining all the conveniences of retroviral vectors. The current improvements in lentiviral vectors eliminate the possibility of regenerating RCR and thus are excellent biosafety measures. However, because there is no animal model for HIV-1 infection, the efficacy and biosafety of lentiviral vectors can only be tested by clinical trials.

One problem associated with both lentiviral and retroviral vectors is the random integration of the transducing vector in the recipient cells. Because integration occurs without sequence specificity, there is the possibility that integration may activate an oncogene or inactivate a tumor suppressor gene. If integration can be targeted to a predetermined site in the chromosome either by modifying the integrase protein or the host factor that binds to it, then the danger of integrating into a harmful site can be eliminated.<sup>38</sup>

The ability to express the transgene from an internal promoter rather than LTRs of the vectors as in the case of lentiviral vectors provides great flexibility in controlling expression of the transgene. For example, liver-specific promoters or cognate promoters, if they are available, can be used to express a liver-specific gene. This ensures that the transgene is expressed at desired physiologic levels.

Unlike for retroviral vectors, no packaging cell lines are yet available for lentiviral vectors. Cell lines that constitutively express *trans*-acting factors eliminate the need for transient transfection of multiple constructs and will allow the generation of the large quantities of virus preparations necessary for *in vivo* gene therapy.

Another flexibility of retroviral vectors is that they can be pseudotyped with envelopes from other viruses, thus expanding the possibility of host range. Chimeric envelopes have been generated to incorporate part of the ligand for a cellular receptor. Viruses pseudotyped with such hybrid envelope proteins then interact with the cell surface receptor dictated by the ligand and mediate the cell type-specific infection by the retroviruses.<sup>39,40</sup> One can envision developing such hybrid envelopes for use in liver gene therapy. The lentiviral or retroviral vectors

could be pseudotyped with chimeric molecules carrying a hepatocyte-specific ligand. Such virus would specifically infect liver cells for efficient gene delivery.

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## ABBREVIATIONS USED

CMV	cytomegalovirus
eGFP	eukaryotic humanized green fluorescent protein
HIV-1	human immunodeficiency virus type 1
LDL	low-density lipoprotein
LTR	long terminal repeat
Mo-MuLV	Moloney-murine leukemia virus
PIC	preintegration complex
RCR	replication-competent recombinant
RES	ribosomal entry site
RRE	Rev response element
SIN	self-inactivating retroviral
TAR	transactivation response
VSV	vesicular stomatitis virus
WHHEL	Watanabe heritable hyperlipidemic

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## Alternative Splice Acceptor Utilization during Human Immunodeficiency Virus Type 1 Infection of Cultured Cells

JOHN C. GUATELLI,<sup>1,2\*</sup> THOMAS R. GINGERAS,<sup>3</sup> AND DOUGLAS D. RICHMAN<sup>1,2,4</sup>

Departments of Medicine<sup>1</sup> and Pathology,<sup>4</sup> University of California, San Diego, and School of Medicine, San Diego Veterans Administration Medical Center,<sup>2</sup> San Diego, California 92161, and the Salk Institute Biotechnology/Industrial Associates, Inc., La Jolla, California 92037<sup>3</sup>

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The utilization of alternative splice acceptors for excision of the 5' major intron of human immunodeficiency virus type 1 RNA was observed after infection *in vitro*. Specific splice events were monitored by a cDNA-polymerase chain reaction. These splice events shared a common splice donor but utilized several alternative splice acceptors. In addition to identifying the previously documented splice acceptors for *tat* and *nef* (S. K. Arya, C. Guo, S. F. Josephs, and F. Wong-Staal, *Science* 229:69-73, 1985), nucleotide sequence analysis of cDNA-polymerase chain reaction fragments also revealed the following: (i) two splice acceptors 15 and 9 nucleotides upstream from the *rev* start codon, which are utilized to create transcripts suitable for specific *rev* expression; and (ii) use of the splice acceptor previously attributed to *nef* to generate a singly spliced, *env*-encoding transcript. Hybridization signals representing the *nef/env*, *tat*, and *rev* splice events increased in intensity between 6 and 12 h after infection of CEM cells with the LAV-1<sub>BRU</sub> strain of human immunodeficiency virus type 1. In contrast, the signal for utilization of the *nef/env* splice acceptor for the singly spliced *env* transcript appeared first at 12 h and increased to maximum intensity by 24 h. The *nef/env* splice acceptor was dominant at all time points examined. We propose that this dominance ensures efficient downstream splicing proximal to the *env* initiation codon in singly spliced transcripts. However, early after infection, the dominance of the *nef/env* splice acceptor appears to divert primary transcripts away from *tat*- and *rev*-specific processing paths. The relative proportions of hybridization signals representing these alternative splice events remained constant throughout the viral replicative cycle. This result suggests that *trans*-acting factors that might influence splice choices are not induced during infection, but rather that *cis*-acting, sequence-specific splice preferences determine the relative efficiency of alternative acceptor utilization.

The genome of human immunodeficiency virus type 1 (HIV-1) encodes multiple structural and regulatory genes of diverse functions; however, viral DNA contains only one transcription initiation site. Differential gene expression must therefore be achieved by posttranscriptional functions: RNA processing, transport, or translational utilization. In this report we examine the possibility that differential gene expression is controlled at the level of gene-specific RNA splice events. The utilization of gene-specific splice acceptors during high-multiplicity HIV-1 infection of the T-lym-phoblastoid cell line CEM (10) has been examined to determine the extent to which alternative splicing provides for temporally ordered gene expression.

Processed RNAs of HIV-1 are, in general, doubly or singly spliced (22). The major 5' intron includes the *gag-pol* sequence and is deleted from singly spliced transcripts. These transcripts are believed to express the structural gene *env* as well as genes *vif*, *vpr*, and *vpu* (22, 23). The 3' intron includes a large portion of the *env* coding region and is deleted from the doubly spliced transcripts believed to express the regulatory genes *tat*, *rev*, and *nef*. These regulatory gene transcripts accumulate before the appearance of singly spliced transcripts after transfection with biologically competent viral DNA (30) and after single-cycle infection of lymphoid cell lines (15). However, differentiation of these RNAs encoding the three regulatory genes has been difficult because of their similar sizes (1.8 to 2.0 kilobases) and shared nucleotide sequences.

The determination of a temporal order of regulatory gene

expression is of interest because these gene products have disparate effects on viral replication. The *tat* gene product is a positive *trans*-activator of viral long terminal repeat-directed transcription and enhances the translatability of such messages (2, 4, 7, 24). The *rev* gene product promotes the accumulation of singly spliced and unspliced RNAs while modestly depressing transcription (7, 8, 19). Both *tat* and *rev* genes are required for viral replication (5, 9, 28). The *nef* gene product has a controversial role as a repressor of transcription (1, 14, 16, 17).

We have exploited the fact that although transcripts encoding the regulatory genes *tat*, *rev*, and *nef* have identical 3' introns, they differ in their utilization of splice acceptors following deletion of their 5' introns (2, 25). At least three splice acceptors are present in a 200-nucleotide region located in the middle of genome. By utilizing primer-directed *in vitro* amplification (polymerase chain reaction [PCR]) (26), it is possible to generate short cDNA fragments that represent the alternative use of these splice acceptors. These fragments are easily resolved by electrophoresis and detected by using oligonucleotide probes, revealing a hierarchy of alternative splice acceptor utilization during HIV-1 infection.

### MATERIALS AND METHODS

**Virus and infection.** The LAV-1<sub>BRU</sub> strain of HIV was propagated in the CD4-positive, lymphoblastoid cell line CEM (10). Viral stocks were prepared by freeze-thaw lysis followed by low-speed centrifugation to remove cellular debris (13). Titers of stocks were determined by a terminal-dilution assay on 96-well microdilution plates containing CEM cells. Infected wells were determined by p24 antigen

\* Corresponding author.

assay (Abbott Laboratories, North Chicago, Ill.) of the culture supernatants at 9 days. For time-course experiments, CEM cells were exposed to a multiplicity of infection of 10.50% tissue culture infective doses per cell for 2 h at 4°C. Cells were warmed to 37°C for 30 min, washed, and then incubated at 37°C. At each time point, samples were taken for nucleic acid purification, determination of cell count and viability, and culture supernatant plus p24 antigen assay. The times indicated in the figures indicate the periods of incubation at 37°C after infection.

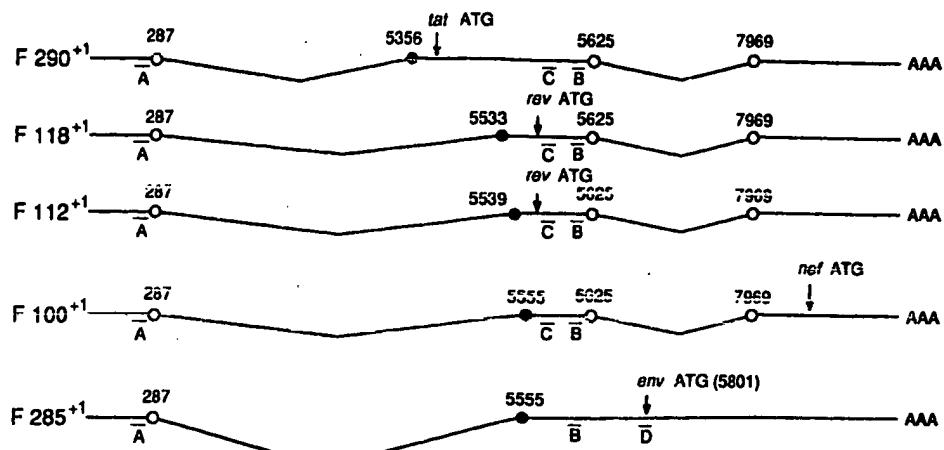
**cDNA amplification and hybridization analysis.** Total nucleic acids were prepared by cell lysis in 0.2% sodium dodecyl sulfate-150 mM NaCl-10 mM EDTA-20 mM Tris (pH 7.5)-200 µg of proteinase K per ml at 50°C for 45 min. Lysates were extracted twice with phenol-chloroform and once with chloroform, adjusted to 0.8 M LiCl, and precipitated with 3 volumes of ethanol. Pelleted nucleic acids were suspended briefly in 10 mM Tris (pH 8.3) and then made up to 100 µl in a solution containing 50 mM Tris (pH 8.3), 10 mM KCl, 2.5 mM MgCl<sub>2</sub>, 10 µg of bovine serum albumin, 0.2 mM each deoxynucleoside triphosphate, and 0.25 µg of each oligonucleotide primer. Nucleic acids were warmed for 90 s at 65°C to denature any RNA secondary structure and then cooled to 42°C for 2 min to anneal primers to target RNAs. Murine-leukemia virus reverse transcriptase (100 U; Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was added, followed by incubation at 42°C for 30 min. The preparations were boiled for 2 min to denature RNA-DNA hybrids and then cooled to 55°C for 1 min before the addition of 2.5 U of *Taq* DNA polymerase (Perkin-Elmer Corp., Norwalk, Conn.). A 30-cycle PCR protocol (26) with primer extension at 72°C for 3 min, denaturation at 94°C for 1 min, and primer annealing at 55°C for 1 min was accomplished in a Perkin-Elmer thermal cycler. This protocol efficiently amplified targets at least 600 base pairs in length. Then 10 µl (10%) of each amplification reaction was applied to a 5% nondenaturing polyacrylamide gel. After electrophoresis, gels were blotted in TBE buffer (20) onto Zeta-Probe nylon membranes (Bio-Rad Laboratories, Richmond, Calif.), and nucleic acids were denatured on the membrane by treatment with 0.4 N NaOH for 30 min and then neutralized with 1 M Tris (pH 8) for 2 to 3 min. Hybridization for 1 h was performed at 55°C in 1% sodium dodecyl sulfate-5×SSPE (20)-0.5% bovine serum albumin-0.5% polyvinylpyrrolidone, including 25 ng of <sup>32</sup>P-end-labeled oligonucleotide probe, followed by washing in 1% sodium dodecyl sulfate-1×SSPE at 55°C. Autoradiography was performed at -70°C with one intensifying screen for 1 to 2 h. Oligonucleotides were prepared with an Applied Biosystems synthesizer and purified with a C<sub>18</sub> column by high-pressure liquid chromatography or were obtained after gel purification from Genetic Designs, Houston, Texas.

**Nucleotide sequence analysis of cDNA fragments.** Nucleotide sequences of specific amplification products were determined by either analysis of plasmid clones or direct analysis of gel-purified cDNA-PCR fragments. For plasmid cloning, primers encoding the identical sequences as A and B but containing 5' extensions encoding *Bam*HI or *Hind*III sites plus 25 additional bases were used to amplify nucleic acids from 10<sup>4</sup> HIV-infected CEM cells. Total cDNA-PCR products from two reactions were pooled, digested overnight with *Bam*HI and *Hind*III, and then gel purified from a 2% agarose gel. DNA fragments of 100 to 150 base pairs were ligated to *Bam*HI-*Hind*III-cut, phosphatase-treated psp6T<sub>19</sub> (Bethesda Research Laboratories). After transformation of *Escherichia coli* MC1061, miniculture plasmid

DNA preparations were used as templates for  $\alpha$ -<sup>35</sup>S incorporation, dideoxy-sequencing reactions (Sequenase protocol; U.S. Biochemical Corp., Cleveland, Ohio). For direct sequencing of cDNA-PCR fragments, 25-µl (25%) samples of the amplification products were electrophoresed on 5% polyacrylamide gels and stained with ethidium bromide, and the specific bands were excised. DNA was passively eluted into TE (20), ethanol precipitated, and then suspended in 25 µl of TE. The concentration of the DNA was estimated by comparative ethidium intensity on agarose gels. Approximately 0.01 fmol of fragment was amplified by using asymmetric PCR (12) to generate single strands as described above, except that 0.5 µg of 5' primer (A) and 0.005 µg of 3' primer (B or D) was used in a 35-cycle protocol using 5 U of *Taq* polymerase, 72°C primer extension was for 2 min, denaturation was at 94°C for 30 s, and annealing was at 55°C for 30 s. This protocol generates an excess of single-strand, plus-sense DNA due to the limiting amount of 3' antisense primer. The products of the asymmetric PCR were purified on Nensorb columns (Dupont, NEN Research Products, Boston, Mass.) and suspended in 15 µl of TE, and 7 µl was used in dideoxy-sequencing reactions as above, except that 1 pmol of sequencing primer was used. Fragments F290, F285, and F100 were sequenced with antisense primer C; F290 was additionally sequenced with an antisense primer (5' GCAATGAAAGCAACACTTTTACAATA 3') that is complementary to a region 5' of the *rev* splice.

## RESULTS

**Characterization of cDNA fragments representing alternative splice acceptor utilization for deletion of the 5' intron.** CEM cells were infected at a low multiplicity and harvested 14 days after infection when maximal activity reverse transcriptase in the culture supernatants was attained (10). Nucleic acids isolated from such late harvests were expected to include mRNAs for all regulatory and structural genes. When cDNA sequences from such cells were amplified by using oligonucleotide primers corresponding to sequences on opposite sides of the 5' splice junctions used to generate processed HIV-1 RNAs, several HIV-specific fragments resulted (Fig. 1). In this experiment, primer A corresponded to the sequence immediately 5' of the splice donor (nucleotide 287); primer B was complementary to the sequence at the 3' end of the first coding exon of the *tat* and *rev* genes. Four cDNA-PCR fragments were characterized; all fragments contained consensus splice junctions upon nucleotide sequence analysis, predicting introns ending in the dinucleotide AG (Table 1). Fragment F290 reflected a splice junction between nucleotides 287 and 5356, identical to that contained in cDNA clone pCV1 (2). Such cDNAs are known to express *tat* and, less efficiently, *rev* (24). Fragments F118 and F112 reflected splice junctions between nucleotides 287 and 5533 and 5539, respectively. These splice acceptors, located 15 and 9 nucleotides upstream from the *rev* start codon, were suitably positioned to generate processed transcripts capable of specifically expressing the *rev* gene while excluding expression of *tat*. Indeed, site-specific mutagenesis of this acceptor region has resulted in a *rev*-deficient phenotype (25). Interestingly, the splice junction sequences depicted by F118 and F112 differed by three nucleotides from that obtained by S<sub>1</sub> nuclease protection mapping (25); however, F118 and F112 sequences appeared to resemble more closely the splice junction consensus (21), predicting introns ending in the dinucleotide AG. Fragment F100 reflected a splice junction between nucleotides 287 and 5555,



**FIG. 1. Strategy and results of alternative splice acceptor detection.** Line drawings represent target mRNAs: +1, cap site; O, splice donor; , splice acceptor. F290, F118, F112, and F100 represent DNA fragments resulting from cDNA-PCR reactions with primers A and B. The splice junctions contained in each fragment were determined by nucleotide sequence analysis. F290 resulted from use of an acceptor at nucleotide 5356; F118 used an acceptor at 5533, F112 used an acceptor at 5539, and F100 used an acceptor at 5555. Oligonucleotide C is a probe for a sequence common to F290, F118, F112, and F100. F285 represents a DNA fragment resulting from cDNA-PCR reactions with primers A and D. F285 used the acceptor at nucleotide 5555. The oligonucleotide sequences used were as follows: A, 5' ACGGCAAGAG GCGAGGGGAGGCGACTG 3'; B, 5' CTTTGATAGAGAACTTGATGAGTCTG 3'; C, 5' CGGAGACAGCGACGAAGACCTCCTCAA GGC 3'; D, 5' CTTCACCTCTCATGCCACTGTCTCTGC 3'.

identical to that contained in clone pCV3(2). Doubly spliced transcripts utilizing this splice acceptor are believed to express the *nef* gene, since the *nef* open reading frame is the only gene fully encoded by this sequence (2).

**Identification of the major splice acceptor utilized for singly spliced transcripts.** The analysis described above reflected the use of alternative splice acceptors suitably positioned to result in differential expression of the *tat*, *rev*, and *nef* genes; however, the 3' ends of the RNAs that yielded these amplification products were not determined. These splice acceptors could potentially be used to generate either doubly or singly spliced transcripts. To define the major splice event leading to singly spliced transcripts, cDNA sequences from HIV-1-infected, late-harvested CEM cells were amplified by using primers A and D (Fig. 1). Primer D spans the *env* start codon, located in the 3' intron of doubly spliced transcripts and can only give rise to amplification products representing singly spliced sequences. The nucleotide sequence of the major fragment, F285, contained a splice junction between nucleotides 287 and 5555, identical to that contained in F100 and, as noted above, previously ascribed to *nef*-expressing

transcripts (Fig. 1, Table 1). The major splice acceptor utilized for singly spliced RNAs, therefore, is identical to the previously proposed *nef* splice acceptor. Singly spliced RNA utilizing this acceptor would encode *vpu* and *env* coding regions. This acceptor is hereafter referred to as *nef/env*.

**Temporal order of alternative splice events during HIV-1 replication.** To determine whether these characterized alternative splice events occurred in an ordered fashion during the HIV-1 replicative cycle, CEM cultures were examined sequentially after infection with the HIV-1<sub>BRU</sub> strain. High multiplicities of infection were used to obtain a synchronous infection. At a multiplicity of infection of 10–50% tissue culture infective doses per cell, cDNA amplification with primers A and C (Fig. 2A) revealed the presence of sequences containing the *nef/env* (F100), *tat* (F290), and *rev* (F118/F112) splice junctions at 1 h postinfection. The *nef/env* splice acceptor was predominantly utilized at this and all subsequent time points. Hybridization signals representing all three splice junctions began to increase between 6 and 8 h and reached maximum intensity at 12 h postinfection. cDNA-PCR with primers A and D (Fig. 2B) revealed initial

**TABLE 1. Nucleotide sequences of cDNA fragments and splice acceptors<sup>a</sup>**

Fragment	Sequence
F290 ( <i>tat</i> )	5' GGGGAGGGCACTG AATTGGGTGTCGACATAGCAGAAATAGCGTTACTCGACAGAGGAGAGCAAGAAATGG 3'
F285 ( <i>env</i> )	5' GGGGAGGGCACTG GAAGAACGGAGAACACAGC 3'
F118 ( <i>rev</i> )	5' GGGGAGGGCACTG CCTTAGGCATCTCCTATGG 3'
F112 ( <i>rev</i> )	5' GGGGAGGGCACTG GCATCTCCTATGG 3'
F100 ( <i>nef/env</i> )	5' GGGGAGGGCACTG GAAGAACGGAGAACATCGAC 3'
Splice acceptor sequences	YnNYAG <sup>W</sup>
<i>tat</i>	TTTATCCATTTCAG AA
<i>rev</i>	TTCACAAAGAAAAG CC and AACGAAAGCCTTAG GC
<i>nef/env</i>	ATCTCCTATGGCAG GA

<sup>a</sup> Portions of the nucleotide sequences of characterized cDNA fragments. The sequences of F290, F285, and F100 were obtained by using asymmetric PCR to generate single-strand copies of the original gel-purified fragments. The sequences of F118 and F112 were obtained from plasmid clones. Splice junctions are indicated by " ". Initiation codons are underlined. The intron-exon boundaries for the *tat*, *rev*, and *nef/env* splice acceptors are shown with the splice acceptor consensus (21). Y indicates pyrimidine, and N indicates any base. The intron sequences for the *rev* and *nef/env* acceptors were obtained in this study and conform to published lymphadenopathy-associated virus sequence (29); the intron sequence for the *tat* acceptor is from the literature (29).

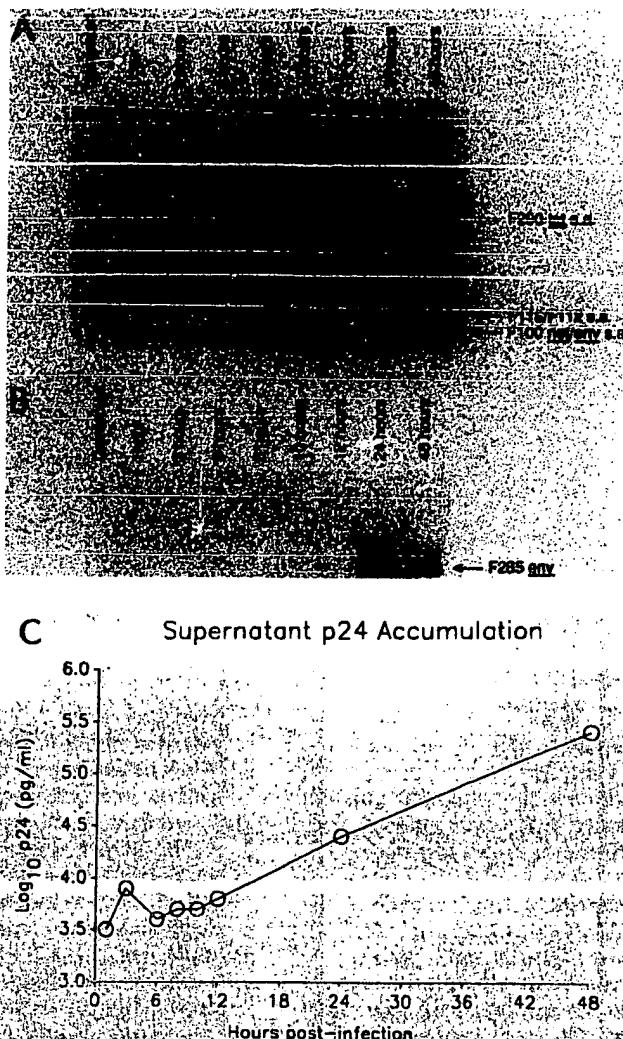


FIG. 2. Kinetics of spliced sequence accumulation and p24 antigen during high-multiplicity infection. CEM cells were infected at a multiplicity of infection of 10 50% tissue culture infective doses per cell. At the indicated times, cells were harvested, and nucleic acids were isolated. (A) cDNA-PCR amplification with primers A and B; nucleic acids from  $10^4$  cells were amplified in each reaction, and 10% of the amplification products were applied to a 5% acrylamide gel. After blotting, membranes were hybridized to oligonucleotide C and exposed at  $-70^{\circ}\text{C}$  for 1 h. (B) cDNA-PCR reactions with primers A and D. Equal amounts of the nucleic acid extracts analyzed in panel A were used. Hybridization was to oligonucleotide B, and exposure time was 2 h. (C) Concentrations of p24 antigen in the supernatants of this culture.

appearance of the *nef/env* splice junction in singly spliced transcripts at 12 h, followed by an increase to maximum intensity by 24 h. The onset of an exponential increase in p24 antigen in the culture supernatant correlated with the appearance of the structural *env* transcript (Fig. 2C).

The splice junctions detected between 1 and 10 h after infection (Fig. 2A) are likely to represent sequences contained in doubly spliced RNAs. This inference is based on the later appearance of singly spliced sequences depicted in Fig. 2B. The detection of spliced sequences at 1 h after infection appeared to be due to their presence in the virus inoculum. Although spliced RNAs should theoretically be absent from the inoculum, low levels of *tat*, *rev*, and *nef/env*

splice junction sequences were detected in the cell-free viral preparation (data not shown). Furthermore, pretreatment of cells with azidothymidine failed to block consistently the appearance of spliced sequences at 1 h after infection (data not shown). Whether these spliced sequences (Fig. 2A) are present as free RNA or are packaged at low efficiency is not clear.

## DISCUSSION

The temporal use of alternate splice acceptors for viral RNA processing during HIV-1 replication has been analyzed. This analysis addresses the question of whether gene-specific alternative splicing defines an order of viral gene expression. To observe closely related alternative splice events occurring in a small number of cells, an assay based upon PCR has been developed by positioning primers for in vitro amplification of cDNAs to encompass alternative splice sites. Each splice event yielded a DNA fragment of a specific size. Several features of this approach merit emphasis. Sequences representing alternative splice events were amplified by using the same primers in the same reaction and were hybridized with a probe that was specific for a common sequence (Fig. 1). Thus, it is unlikely that the different signal intensities for the alternatively spliced sequences are attributable to differential amplification or hybridization efficiencies.

HIV-1 pre-mRNA splicing is complicated by the use of multiple splice donors and acceptors (2, 22, 23). The use of these sites generates both multiple transcripts capable of expressing the same gene and polycistronic transcripts theoretically capable of expressing several genes. By restricting our analysis to the utilization of alternative 5' splice acceptors, however, the interpretation of the data derived by cDNA-PCR can be simplified.

The *tat* gene, for example, can be expressed from doubly spliced transcripts such as those represented by clone pCV1 (2), from singly spliced transcripts producing a truncated but functional protein due to an in-frame termination codon (18), or from a transcript containing an additional exon encoding part of the *env* gene sequence (*tat/env* gene) (27). Yet all these transcripts utilize the splice acceptor at nucleotide 5356 and are represented by fragment F290 (Fig. 1). The importance of this splice site was also noted when this acceptor region was deleted from a genomic viral DNA clone: the clone did not support *trans* activation and supported only greatly reduced viral production (9).

In the case of *rev*, expression could conceivably result from translation of a polycistronic transcript utilizing the acceptor at nucleotide 5356 (Fig. 1). However, a point mutation 5 nucleotides downstream from the splice acceptor contained in F118 (Fig. 1) has been shown previously to eliminate full-length viral RNA production and require a *rev* expression vector for complementation; S1 nuclease analysis predicted a splice acceptor 3 nucleotides downstream of the F118 acceptor (25). It is of interest that the mutation causing partial *rev* deficiency involved an AG dinucleotide 6 base pairs 3' of the AG reported for F118. This is the AG that defines the 3' end of the intron predicted by F112 (Table 1). Careful examination of F118/F112 band reveals that it comprises a doublet of bands of similar intensity (data not shown). It is likely that both acceptors are used for *rev* RNAs, perhaps explaining the incomplete *rev* deficiency obtained by mutation at the more 3' AG alone.

The finding that the splice acceptor at nucleotide 5555 (*nef/env*) was the dominant acceptor used for singly spliced

transcripts (Fig. 2B) is in agreement with the reported S1 analysis of a mutant genomic clone that expressed only 1.8- and 4.3-kilobase RNA (25). The presence of amplification products larger than F285 indicates that acceptors further 5' are used as well (Fig. 2B).

Alternative splice events have been reported in addition to those characterized in the present report (22, 23). The multitude of DNA fragments resulting from the cDNA-PCR analysis (Fig. 2A) also suggests that many additional 5' splice acceptors are used. However, the data reviewed above support the contention that F290 represents *tat* RNA, F118/F112 *rev* RNA, and F285 *env* RNA. However, F100 present early after infection may not represent fully *nef* RNAs. Although it is likely that F100 signals appearing earlier than 24 h after infection originate from doubly spliced transcripts, a novel cDNA has recently been reported which represents a direct single splice from the 5'-most splice donor to the 3'-most splice acceptor in the viral genome (27). Such a transcript would also express *nef* and cannot be detected by the cDNA-PCR assays reported here.

The temporal study of alternative splicing during HIV-1 infection (Fig. 2) revealed low levels of *tat* (F290), *rev* (F118/F112), and *nef/env* (F100) splice junction sequences as early as 1 h after infection. These early signals representing spliced sequences were not sensitive to azidothymidine and were likely the result of residual sequences from the inoculum preparation. At 6 to 8 h after infection, the levels of these spliced sequences began to increase; this increase marked the onset of new viral RNA synthesis. The onset of accumulation of singly spliced structural gene (*env*) transcripts (F285) occurred an additional 6 h after the early RNAs, beginning at about 12 h after infection. This delay in the appearance of structural gene transcripts has been shown previously by Northern RNA blot analysis (15, 30). The accumulation of p24 antigen in the culture supernatant was coincident with the increase of *env* transcripts. These observations are consistent with the hypothesis that the appearance of singly spliced transcripts for structural polypeptides must follow the appearance of the *rev* protein.

In this CEM cell system and with the viral strain HIV-1<sub>BRU</sub>, gene-specific splice acceptor utilization does not define a clear order of regulatory gene expression. Low but definite levels of each regulatory gene-specific splice were detected at the earliest assayed time point. Furthermore, the relative intensities of the hybridization signals for the characterized splice events remained fairly constant over time (Fig. 2A). This result implies that, rather than a switch over time from one splice site to another as might be regulated by cellular or viral *trans*-acting factors (3, 11), the relative amounts of alternatively spliced sequences are more likely determined by *cis*-acting, sequence-specific splice preferences (6).

A relative hierarchy of utilization of the *tat*, *rev*, and *nef/env* acceptors is apparent, however. The data clearly indicate that the *nef/env* splice acceptor is dominant compared with the acceptors for *tat* and *rev* (Fig. 2A). This dominance of the *nef/env* acceptor is seen with two distinct primer sets (AB and AD amplifications; Fig. 2A and B, respectively); this observation weighs against the possibility that this finding is an artifact of the amplification assay. Furthermore, the *nef/env* splice acceptor sequence seems to fit more closely the consensus sequence when compared with the *tat* and *rev* acceptors (Table 1) (21). In singly spliced transcripts, dominance of the *nef/env* acceptor would ensure relatively efficient downstream splicing more proximal to the *env* initiation codon. However, early after infection this

dominance appears to favor *nef* splicing, diverting primary transcripts away from *tat* and *rev* expression. The viral genome appears to be organized such that expression of the essential positive regulatory genes *tat* and *rev* is dependent on utilization of relatively weak splice acceptors located 5' of the more efficient *nef/env* splice acceptor. This arrangement may provide a mechanism for the virus to sense the host-cell transcriptional environment, delaying activation of its own positive regulatory mechanisms until this environment is optimal for replication.

The extension of the methods of analysis described here to other cells and viral isolates may reveal different alternative splice preferences. For example, we have found that primary human macrophages infected with the HTLV-III<sub>BALB/c</sub> strain of HIV-1 utilize the *tat* splice acceptor at nucleotide 5356 less efficiently than CEM cells infected with HIV-1<sub>BRU</sub> (J. Guatelli and J. Munis, unpublished data). Such differences may be important in understanding the characteristics of HIV-1 replication in different host cell types. The present study serves as a base line for such comparisons.

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#### ADDENDUM IN PROOF

An extensive analysis of HIV-1 mRNA splicing has recently been reported which includes a description of the two *rev* splice acceptors described here (S. Schwartz, B. K. Feilker, D. M. Benko, E. M. Fenyo, and G. N. Pavlakis, *J. Virol.* 64:2519-2529, 1990).

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## Residual expression of reporter genes in constructs mimicking HIV genome organization

N. Chenciner <sup>(1)</sup>, L. Pedroza Martins <sup>(1)</sup>, N. Hanania <sup>(2)</sup>, L. Stratford Perricaudet <sup>(2)</sup>,  
M. Perricaudet <sup>(2)</sup> and S. Wain-Hobson <sup>(1)</sup> <sup>(\*)</sup>

<sup>(1)</sup> Unité de Rétrovirologie moléculaire, Institut Pasteur, 75724 Paris Cedex 15, and <sup>(2)</sup> Laboratoire de Génétique des Virus oncogènes, Institut Gustave Roussy, PRII, 94805 Villejuif Cedex (France)

### SUMMARY

Plasmids were constructed whereby the expression of a reporter gene, either the cDNA corresponding to the secreted form of human alkaline phosphatase (SEAP) or the herpes simplex virus type 1 (HSV1) thymidine kinase (tk) gene, was rendered dependent upon the expression of the human immunodeficiency virus type 1 (HIV1) tat and rev proteins. The SEAP or tk genes were placed between HIV1 splice donor and acceptor sites. One SEAP construct carried a series of alternating splice donor and acceptor sites. In all cases, the rev response element mapped within an intron. Despite such mimicry of the HIV1 genome, residual expression of the reporter gene in the absence of tat and rev was observed. These results, as well as non-specific T-cell recruitment, suggest limits to the specificity of using HIV-activated toxic gene expression to kill HIV-infected cells.

**Key-words:** HIV, Protein tat, Protein rev, Gene SEAP, Gene tk; Cytotoxicity, Reporter genes, Gene therapy.

### INTRODUCTION

The regulation of expression of the human immunodeficiency viruses (HIV) is complex and depends on two virally encoded transacting proteins, tat and rev. Both act on RNA tertiary structures; tat acts upon the transactivation response (TAR) element located within the R region of the LTR, while rev interacts with the rev response element (RRE) which maps to within the gp41 transmembrane envelope-coding region.

Tat is pleiotropic but overall acts as an ampli-

fier of HIV transcription, binding to the TFIID elongation factor (Kashanchi *et al.*, 1994). Transcription from the LTR follows T-cell activation, giving rise to small mRNA transcripts encoding tat, rev and nef yet devoid of the RRE. The newly synthesized tat is translocated to the nucleus where, through interaction with TAR, it results in massive amplification of proviral expression. Rev temporally coordinates the expression of HIV mRNAs. Beyond a certain threshold concentration, rev acts upon partially or unspliced RRE-containing mRNA ensuring transport to the cytoplasm and subsequent translation (Feinberg *et al.*,

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<sup>(\*)</sup> Corresponding author.

1986; Sodroski *et al.*, 1986; Felber *et al.*, 1989; Pavlakis *et al.*, 1990).

Quite how this is achieved is not settled. Certainly, rev shuttles back and forth between the nucleus and cytoplasm via the nucleolus and binds specifically to a human nucleolar shuttle protein (Fankhauser *et al.*, 1991). Clearly rev function requires RRE (Malim *et al.*, 1989; Hadzopoulou-Cladaras *et al.*, 1989; Hammarskjöld *et al.*, 1989; Nasoulias *et al.*, 1994; Staffa *et al.*, 1994). What is less clear is to what extent other sequences, splice junctions (Chang and Sharp, 1989), cis-acting repressor sequences (CRS) located within the env gene upstream of the RRE (Rosen *et al.*, 1988; Nasoulias *et al.*, 1994) and others in gag are also necessary (Schwartz *et al.*, 1992).

These two complex, yet HIV-specific systems, have been used to express heterologous cytotoxic genes. Among tat-dependent constructs are the poliovirus 2A protein (Sun *et al.*, 1989) or  $\alpha$ 2-interferon (Bednarik *et al.*, 1989). By exploiting both the tat/TAR and rev/RRE elements, the diphtheria toxin A gene (DT-A; Harrisson *et al.*, 1991) or herpes simplex virus thymidine kinase gene (HSV1, tk; Venkatesh *et al.*, 1990; Caruso *et al.*, 1992) were used to selectively kill cells expressing HIV1 tat and rev. Tk expression may be toxic for cells in the presence of nucleoside analogues such as acyclovir (Furman *et al.*, 1980). In these studies, the reporter/toxic genes were not flanked by splice sites.

Here, plasmids were constructed in which the highly sensitive SEAP reporter gene or the HSV1 tk was placed between HIV1 splice donor and acceptor sites, thus mimicking the organization of many HIV1 ORFs. Despite this, a recurrent basal expression of the reporter genes was noted, suggesting limits to their use in AIDS gene therapy.

## MATERIALS AND METHODS

### Construction of expression vectors

pLTR-RRE was first constructed which would allow subsequent subcloning of both the SEAP and tk reporter genes. It contains a 470-bp fragment encoding the HIV1 LTR upstream of a 722 bp fragment encoding the HIV1 RRE and major tat/rev splice acceptor (A7; Muesing *et al.*, 1985; Schwartz *et al.*, 1990). The LTR and RRE fragments were amplified by PCR from pLAI2 plasmid (Peden *et al.*, 1991) with the following primers: LTR (bases 321-790; Wain-Hobson *et al.*, 1985) 5'-GGAGGATCCAAGAACTGCTGACATCGA and 5'CGAGTCGACTCTCCTCTAGCCTCC; RRE (bases 7749-8471) 5'CCAGTCGACAT-TAGGAGTAGCACCCAC and 5'CTGTTAACATTCCCTCGGGCCTGTCGG; *Bam*H1, *Sal*1 and *Hpa*1 restriction sites are underlined. After purification on acrylamide gels, fragments were subcloned at the *Bam*H1-*Hpa*1 sites of a pML-derived vector containing the early polyadenylation sites of SV40, the two fragments being ligated via the *Sal*1 site introduced into the PCR primers.

The HSV1 tk gene was PCR-amplified as a 1300-bp fragment using primers AD3: 5'-CCAGTCGACTCCCGCACCTCTTG and AD4: 5'CGAGTCGACGTCATAGCGCGGGTTCC, digested by *Sal*1 and inserted into the corresponding site of pLTR-RRE giving rise to plasmid ptk. The 1913-bp *Sal*1-*Xba*1 fragment of the pBC12/pL/SEAP vector (Berger *et al.*, 1988), which encodes the entire SEAP cDNA, was cloned into the *Sal*1 site of plasmid pLTR-RRE. This construct was designated p14. Construct p1713 was derived from p14 by addition at the *Sal*1 site of a fragment encoding HIV1 exon 5 including the tat/rev splice donor (D4) and env/vpu acceptor (A5) sites (Schwartz *et al.*, 1990) amplified from pASArev (Pedroza-Martins *et al.*, 1991). p19'3 was derived from p1713 by deletion of the internal *Hind*III fragment.

### Transfection and SEAP assays

Cells were cotransfected by SEAP plasmids, pSV2tat-Lai (Meyerhans *et al.*, 1989) and pASArev (Pedroza-Martins *et al.*, 1991). Human SW480 cells were transfected by the calcium phosphate

CRS	= cis-acting repressor sequence.
HSV	= herpes simplex virus.
LTR	= long terminal repeat.
ORF	= open reading frame.
RRE	= rev response element.

SDS	= sodium dodecyl sulphate.
SEAP	= secreted form of alkaline phosphatase.
SV40	= simian virus 40.
TAR	= trans activation response.
tk	= thymidine kinase.

method (Chen and Okayama, 1987). CEM-tat and HUT78-tat cells were transfected by using the DEAE-dextran procedure (Fujita *et al.*, 1986). Sixty-hours post-transfection, culture supernatants were cleared by centrifugation and heated for 10 min at 65°C to inactivate any endogenous alkaline phosphatase. SEAP activities were determined at 405 nm in the linear range on 20 µl or 50 µl of supernatant as previously described (Berger *et al.*, 1988).

#### Transfection and tk assays

Mouse Ltk<sup>-</sup> cells were transfected by tk plasmids with and without pSV2tat-Lai and pASArev by the calcium phosphate method. Cultures were grown in HAT medium for 48 h post transfection (Szybalska and Szybalski, 1962). Clones were counted after six days of selection. Some clones were expanded and maintained in the presence of 12 µM of acyclovir [9-(2-hydroxyethoxy-methyl)guanine] selection. Mock-transfected Ltk<sup>-</sup> cells were maintained under identical conditions without acyclovir as controls. After 4 days, viable cells were counted.

#### Isolation and Northern blot analysis of RNA

Total cell RNA was isolated from cells at 72 h post-transfection by the RNAZol B (Bioprobe) procedure (Chomczynski *et al.*, 1987). RNA was electrophoresed in formaldehyde-agarose gel (2%), and transferred to "Hybond N" membrane. Blots were prehybridized for 2-3 h at 42°C in 20 ml of 5 × SSC, 50 mM sodium phosphate pH 6.5, 50% deionized formamide, 0.2% sodium dodecyl sulphate (SDS), 5 × Denhardt solution and 100 µg/ml denatured, sonicated salmon sperm DNA. Hybridization was performed overnight under the same conditions with 25-50 ng of random-primed <sup>32</sup>P-labelled DNA probe. The 470-bp LTR fragment used for probing specific mRNA was purified on acrylamide gel. Blots were briefly washed 3 times in 3 × SSC, 0.1% SDS at room temperature and 1 h in the same solution at 50°C, then 1 × 30 min in 0.1 × SSC, 0.1% SDS at 50°C.

#### RESULTS

##### Construction of HIV1-dependent SEAP and tk vectors

The reporter genes (SEAP or tk) were first cloned 3' to the HIV1 LTR and major splice donor sequence (D1), yet 5' to a 722-bp fragment

encompassing the HIV1 RRE and major tat/rev/nef splice acceptor site (A7, fig. 1). Plasmid p1713 represents a more complicated construction with alternating splice donor and acceptor sites, *i.e.* (D1, A5, D4, A7) due to the introduction of a fragment encoding the vpu/env acceptor sequence (A5) and the tat/rev splice donor (D4). Thus, there is the possibility of creating multiply spliced mRNAs typical of most early HIV1 transcripts. Plasmid p19'3 differed from p1713 by deletion of an internal *Hind*III site which eliminated the D1 splice site (fig. 1).

#### Expression of reporter plasmids expressing SEAP

Reporter gene expression was examined in transient expression assays by calcium phosphate-mediated transfection of SW480 colon carcinoma cells. Each of the three constructs was cotransfected with and without HIV1 tat (pSV2tat) or rev (pASArev) plasmids. In all cases, SEAP expression was increased ~ 6-10-fold by coexpression with tat, which was further augmented ~ 2-3-fold when rev was added (fig. 2). The basal activity of SEAP was significantly positive relative to controls (ptk-transfected cells), *i.e.* the 2-10% of a tat-specific response and 1-5% of a tat + rev-specific response. Stably expressing tat derivatives of the HUT78 and CEM (HUT78-tat and CEM-tat kindly provided by Dr. Jon Karn) were transfected with p19'3 ± pASArev plasmid. The effect of adding rev was approximately two-fold (fig. 3).

#### Expression of the reporter plasmid expressing tk

To determine whether the SEAP gene itself was somehow responsible for the basal level of expression, a plasmid analogous to p14, yet expressing the HSV1 tk gene, was constructed (denoted ptk, fig. 1). Mouse Ltk<sup>-</sup> cells were cotransfected by ptk with or without tat and rev. Forty-eight hours post transfection, cells were selected in HAT medium (Szybalska and Szybalski, 1962). Between 20-30 HAT-resistant clones were obtained per 10<sup>6</sup> cells in the

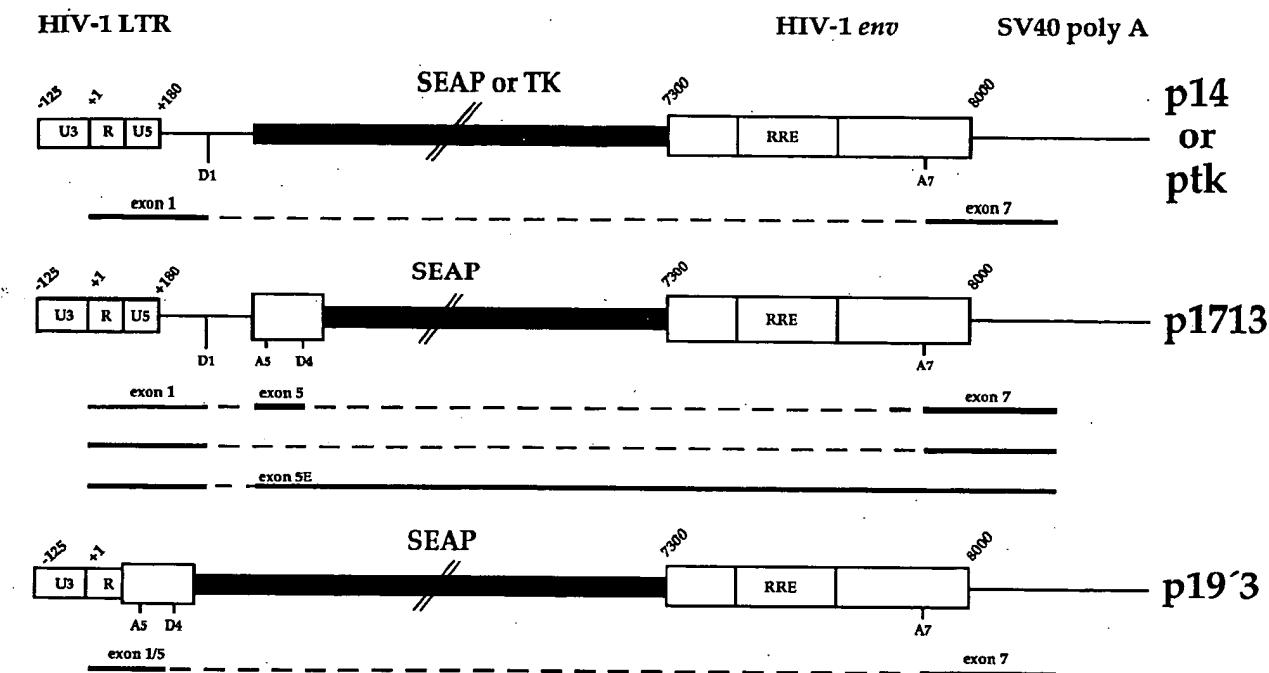


Fig. 1. Tk and SEAP expression plasmids used in expression assays.

The HIV1 LTR includes the enhancer, promoter and the tat responsive element TAR. The env fragment containing RRE region was inserted downstream to the reporter gene. All plasmids included the SV40 polyadenylation signal. In tk and SEAP (p14; p1713) plasmids, the major splice donor site (SD) was introduced before the reporter gene. HIV1 exon 7 with its splice sites was introduced 5' to the SEAP gene, giving rise to plasmid p1713. Plasmid p19'3 was derived from p1713 after deletion of the internal fragment *Hind*III containing the major splice donor site. Exons expected were designated according to Schwartz *et al.* (1990).

absence of tat and rev, while 100-120 clones were obtained when ptk was cotransfected with both tat and rev expressing plasmids. Tk<sup>+</sup> clones derived in the absence of tat + rev, as well as a line derived from the ensemble of clones, were tested for acyclovir-mediated cytotoxicity. A 30-50% reduction in the number of viable cells was noted after four-day treatment with 12  $\mu$ M of acyclovir, a concentration devoid of non-specific toxic effects in untransfected cells.

#### mRNA expression

The modulation of mRNA expression from SEAP and tk reporter plasmids by tat and rev was examined in transient expression assays in human SW480 cells by analysis of total mRNA (fig. 4). For the three SEAP constructions (p14, p1713

and 19'3), no specific mRNAs were detected in the absence of tat even though basal levels of SEAP activity could be detected by functional assays. In the presence of tat, unspliced and spliced RNAs of predicted sizes were detected after transfection. Spliced mRNA of p19'3 was ~100 nucleotides smaller than the spliced p14 mRNA, while two small mRNAs were detected for p1713. In the presence of rev, the proportion of spliced mRNA decreased while the SEAP activity increased two-fold. For the ptk construct, the results were qualitatively similar.

#### DISCUSSION

Tat- and rev-dependent expression vectors are described in which the SEAP or tk reporter gene was placed within an intron, thus reflecting the

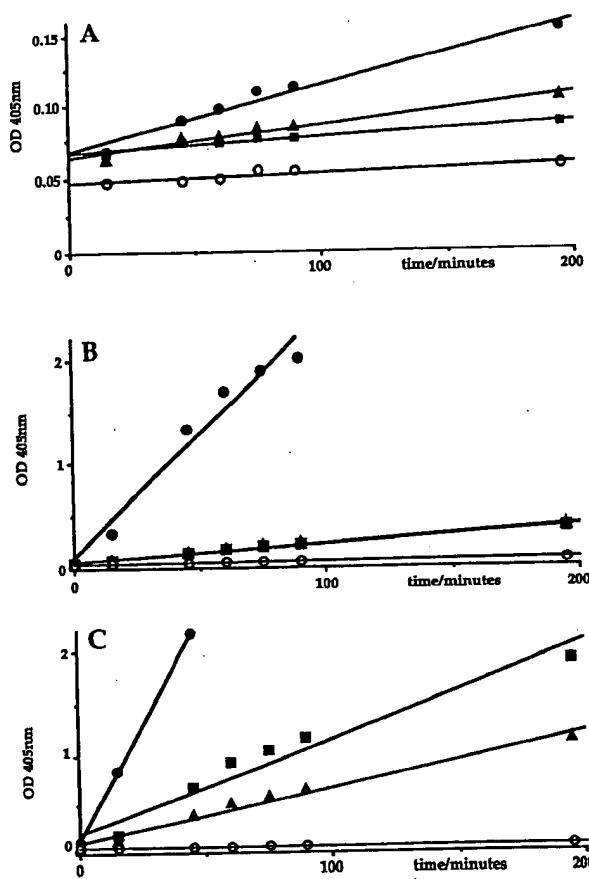


Fig. 2. Time course of SEAP expression (OD 405 nm) as a function of transactivation by tat and rev.

Human SW480 cells were cotransfected with plasmids SEAP (5 µg), pSV2 tat Lai (1 µg) (Meyerhans *et al.*, 1989) and ± pASAre (3 µg) (Pedroza *et al.*, 1991) by the calcium phosphate method. SEAP production was monitored 60 h later in the supernatant as previously described (Berger *et al.*, 1988).

A, basal SEAP expression; B, with pSV2 tat; C, with pSV2tat + pASAre; ● p19'3, ▲ p1713, ■ p14, ○ ptk.

structure of all rev-dependent mRNAs. The order or complexity of splice sites (D1, gene, A7; D1, D4, gene, A7; D4, gene, A7) only slightly changed the effect of tat + rev (figs. 1-4). That the tat/rev 3' acceptor splice site (A7) was recently reported to be rather inefficiently spliced, may help explain why the mRNA was only partially spliced (Staffa *et al.*, 1994).

HIV-regulated expression of a HSV1 tk gene vector has been proposed to target drug-depen-

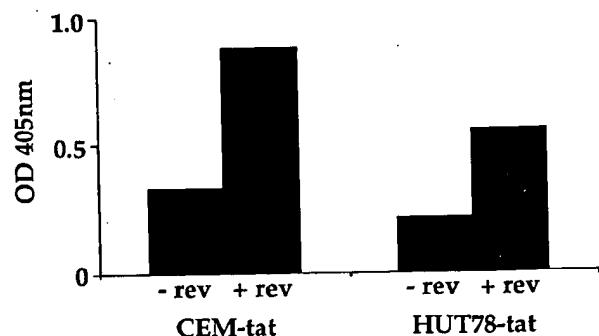


Fig. 3. Rev-dependent SEAP activity in stably transfected tat cell lines.

CEM tat and HUT78 tat cells were transfected by p19'3 + pASAre using the DEAE-dextran procedure. SEAP analyses were made at 24 h because SEAP secretion was very inefficient in these cell lines. The results represent the averages of three experiments.

dent toxicity to HIV-infected cells (Venkatesh *et al.*, 1990; Caruso *et al.*, 1992), however, their attempt to impose rev control was unsuccessful. Other investigators have described the targeting of the diphtheria toxin to HIV-infected cells (Harrison *et al.*, 1991) with tat and rev dependence. Basal expression, while not zero, was reduced by the inclusion of negative regulatory (CRS) sequences from the env gene in the 3' untranslated region of their reporter gene (Harrison *et al.*, 1991). In these three studies, the reporter/toxic gene was not surrounded by splice donor and acceptor sites which should eliminate the gene in the absence of tat and rev. Thus, even when the reporter/toxic gene is properly flanked by HIV1 splice donor and acceptor sites in addition to the TAR and RRE sequences, a residual expression from the HIV1 LTR persisted. The same findings were made from experiments on both human and mouse cells, or with transiently or stably transfected cells.

Human T cells are activated by MHC class II antigen presentation, superantigens and cocktails of cytokines (Unutmaz *et al.*, 1994). The latter is particularly important as the number of activated T cells within a lymph node is out of all proportion to the number of antigen-specific T cells (Doherty *et al.*, 1994). Thus antigen-specific recruitment results in non-specific T-cell activa-

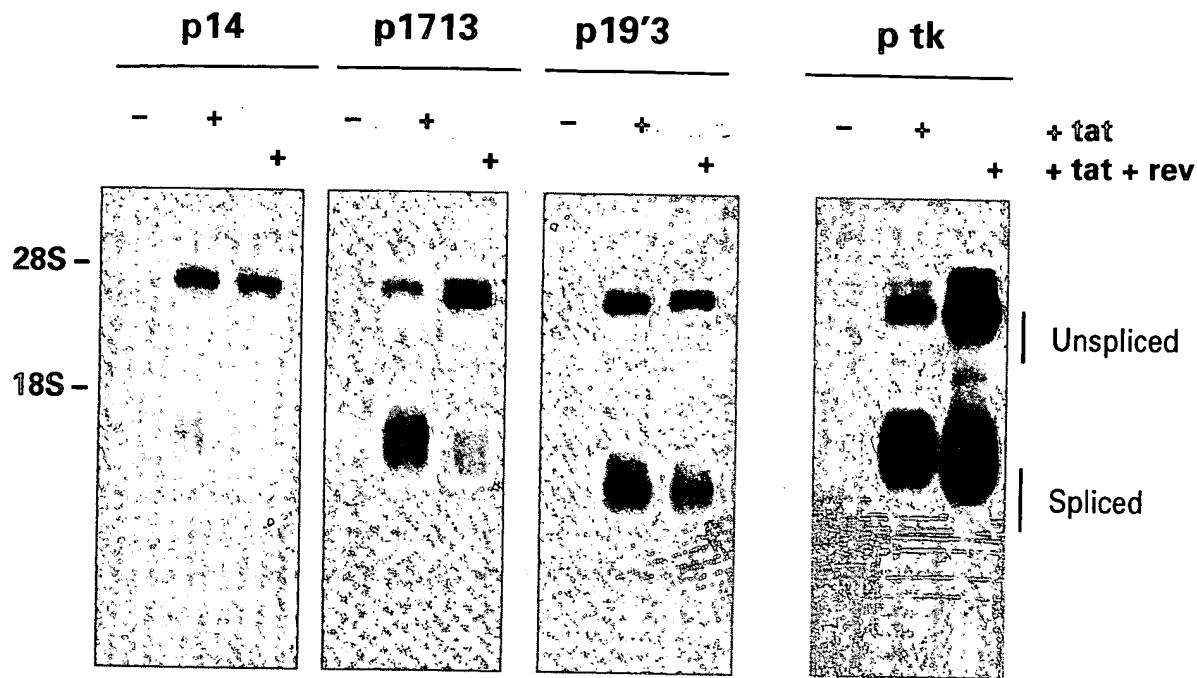


Fig. 4. Northern blot analysis of SEAP- and tk-related transcripts.

SW480 cells were cotransfected with plasmids SEAP and tk (5 µg) and vectors expressing tat (1 µg) or rev (3 µg) as described in figure 2 legend; 20 µg samples of total RNA were analysed by agarose gel electrophoresis and hybridized to random-primed  $^{32}$ P-labelled DNA probe LTR. The lengths of RNA was estimated relative to 28S (4.5 kb) and 18S (1.8 kb) RNA markers. Position of unspliced and spliced RNA are indicated.

tion. The HIV LTR is sensitive to T-cell antigen-dependent and independent activation or treatment with certain cytokines (Tong-Starksen *et al.*, 1987; Nabel *et al.*, 1988; Brinchmann *et al.*, 1992; Janoff *et al.*, 1993; Wallis *et al.*, 1993). In addition, a number of viral regulatory proteins such as adenovirus E1A, cytomegalovirus ICP0 and the HTLV tax protein (Mosca *et al.*, 1987; Davies *et al.*, 1987; Ostrove *et al.*, 1987; Rice *et al.*, 1988) are capable of trans-activating the HIV1 LTR. Thus in the context of HIV-dependent toxic gene therapy, non-specific activation of T cells by the immunological milieu (Unutmaz *et al.*, 1994; Doherty *et al.*, 1994) or other viral coinfections along with residual expression from the HIV1 LTR would probably result in a large number of non-infected T cells being killed. Therefore, the effectiveness of a reagent requiring tat and rev may be called into question. It is possible that, in late stage disease, when cellular immunity is waning, HIV-dependent toxic gene

therapy may be of some use. However, during the long clinically asymptomatic phase, there would appear to be certain limitations to their use.

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#### Expression résiduelle de gènes rapporteurs dans des constructions mimant l'organisation du génome du VIH

Nous avons construit des plasmides porteurs de gènes rapporteurs, soit l'ADNc correspondant à la phosphatase alcaline humaine sous sa forme sécrétée

(SEAP), soit le gène codant pour la thymidine-kinase (tk) du virus herpès simplex type 1 (HSV1) dont l'expression est sous la dépendance des protéines tat et rev du virus de l'immunodéficience humaine de type 1 (VIH1).

Les gènes SEAP et tk sont placés entre les sites donneurs et accepteurs d'épissage du virus VIH1. Dans un plasmide, le gène SEAP est placé entre des sites d'épissage donneurs et accepteurs alternatifs. Dans toutes les constructions, l'élément de réponse à la protéine rev (RRE) est introduit dans un intron. Malgré une telle organisation mimant celle du génome du VIH, une expression résiduelle des gènes rapporteurs en l'absence des protéines tat et rev est observée. Ces résultats, ainsi que le recrutement non spécifique des cellules T, montrent les limites de l'utilisation d'un gène toxique dont l'expression est activée par les protéines de régulation du VIH pour détruire les cellules qu'il infecte.

**Mots-clés:** VIH, Protéine tat, Protéine rev, Gène SEAP, Gène tk; Cytotoxicité, Gènes rapporteurs, Thérapie génique.

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Key words: *visna virus/rev gene/S1 mapping*

## A Transcriptional Map of Visna Virus: Definition of the Second Intron Structure Suggests a *rev*-like Gene Product

By D. R. SARGAN\* AND I. D. BENNET

Department of Veterinary Pathology, University of Edinburgh, Summerhall,  
Edinburgh EH9 1QH, U.K.

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### SUMMARY

Visna virus is the prototype lentivirus, with a genome structure similar to that of the human immunodeficiency viruses HIV-1 and HIV-2. We have analysed *in vitro* the transcription pattern of this virus in lytic infections of choroid plexus cells. Northern blot analysis shows the presence of spliced subgenomic mRNA species of 4.9, 4.3, 4.0, 1.7 and 1.4 kb. Use of appropriate subgenomic probes shows that the first three of these species encode envelope protein (but also potentially the small open reading frames Q and S). The 1.7 kb RNA could contain S. In order to elucidate the translational coding potential of the smallest RNA, and to characterize further all the transcripts, S1 mapping was performed across those parts of the genome which were close to exon/intron boundaries. This allowed the definition of acceptor splice sites following both introns 1 and 2 as well as donor sites preceding intron 2. The data suggest that the 1.4 kb RNA encodes a protein derived from the F reading frame that may form part of a precursor protein and also contains sequences with some degree of homology to the *rev* (*trs/art*) protein of HIV, as well as a typical protease cleavage site between these sequences and the F protein.

### INTRODUCTION

Visna-maedi virus is the prototype virus of the *Lentivirinae*, a subfamily of the *Retroviridae* which includes the human immunodeficiency viruses HIV-1 and HIV-2, equine infectious anaemia virus, caprine arthritis-encephalitis virus and a number of more recently discovered viruses of primates, and domestic and farm animals (reviewed in Haase, 1986). The pathogenesis of the virus-induced diseases is typified by long incubation periods and slow progressive symptomalogies, with death occurring many months or years after infection. In contrast when these viruses are grown in permissive cells in tissue culture the virus multiplies in a comparatively rapid manner leading to cell death within days or weeks. In the case of visna virus it is clear that restriction of viral transcription and translation exists *in vivo*, but that this does not occur *in vitro*; cells containing small numbers of viral transcripts but expressing no detectable quantities of viral antigens are found in the animal, but such cells have not been reported in infections *in vitro* (Brahic *et al.*, 1981; Gendelman *et al.*, 1985).

The lentiviruses are distinguished from other retroviruses by several features of their genome structure including the presence of several small open reading frames (ORFs) in addition to the three structural genes *gag*, *pol* and *env* which are characteristic of the retroviruses (Fig. 1). The functions of these ORFs have been most fully characterized for HIV-1. Transcriptional mapping of this virus has shown that at least two of these reading frames encode specific RNA products in tissue culture (reviewed in Chen, 1986). One of these, *tat III*, encodes a protein essential for efficient replication of the virus which acts both at transcriptional and translational levels (Peterlin *et al.*, 1986; Rosen *et al.*, 1986). The second ORF, *rev* (also called *trs* or *art*), encodes a protein which regulates the production or utilization of the longer viral transcripts, although the mode of action of this protein is not yet fully understood (Feinberg *et al.*, 1986; Sodroski *et al.*, 1986). Mutational analyses of HIV have shown that a third ORF (*vif*) is also

required for the production of fully infectious virus *in vitro* (Fisher *et al.*, 1987). At least three more ORFs are evolutionarily conserved and encode proteins capable of eliciting antibody responses in infected individuals but no function has yet been described for these.

To understand the slow progression of lentivirus-induced diseases requires knowledge of how transcription and expression of viral protein are regulated both in lytic infections in tissue culture and in the restricted type of infection observed as the predominant infection *in vivo*. In lytic infections of choroid plexus fibroblasts, a complex set of visna virus transcripts has been described (Davis *et al.*, 1987; Vigne *et al.*, 1987). These studies showed that visna virus produces, in addition to an unspliced genomic transcript encoding *gag* and *pol* gene products, three spliced transcripts encoding *env* and two smaller messages which are probably doubly spliced. The splice donor junction of the first intron has already been mapped, as have some acceptor sites of this intron (Davis *et al.*, 1987; Vigne *et al.*, 1987). Here we confirm and extend these reports by using S1 nuclease mapping to assign all the major splice junctions of the second intron, and to investigate the major splice acceptor sites of the first intron. This has confirmed the utilization of a *tat III*-like ORF (designated *S* by Sonigo *et al.*, 1985), and the presence of a second intron, and has also suggested the presence of a *rev*-like protein produced as a part of a polyprotein from the F reading frame.

#### METHODS

**Viruses and cell culture.** Visna virus strain 1514 was grown in ovine choroid plexus cells (Weybridge cell line, WSCP) between passages 15 and 30. For maintenance of virus stocks infections of low multiplicity were used. Tissue culture media [Dulbecco's MEM (DMEM) containing 1% foetal calf serum] were harvested for use as a source of virus at various times after infection. For experimental infections, an m.o.i. of approx. 0.1 was used (as judged by titration of the virus in a c.p.e. assay). Supernatants containing virus were clarified by centrifugation (10000 g for 15 min) and incubated with cell monolayers for 1 h at 4 °C followed by 1 h at 37 °C. The monolayers were extensively washed in Hanks' buffered saline solution (HBSS) prior to incubation in DMEM containing 1% foetal calf serum for 72 h or as stated in the figure legends. In general,  $2 \times 10^6$  cells were used as a source of RNA.

**RNA preparation and blots.** RNA was prepared by a modification of the guanidinium thiocyanate method (Sargan *et al.*, 1986). For Northern blotting analysis, 5 or 10 µg samples of the RNA were fractionated by electrophoresis on 1 or 1.2% agarose gels containing 2.2 M-formaldehyde (6.7% v/v) and 20 mM-sodium phosphate pH 7.0. Capillary blotting, hybridization and wash conditions have been described (Sargan *et al.*, 1986).

**Probe preparation and labelling.** A DNA clone of the long *SacI* fragment (8.5 kb) of visna virus strain 1514 was a kind gift of Dr J. E. Clements (Molineaux & Clements, 1983). This was used as a source of restriction fragments from which subclones of the viral genome were derived (see Fig. 1 and legends to other figures). Some of the fragments were purified by two electrophoretic separations through agarose gels and used directly to produce probes. For Northern blots, probe fragments were  $^{32}\text{P}$ -labelled by oligonucleotide-directed labelling (Feinberg & Vogelstein, 1983) to a specific activity of  $> 3 \times 10^8$  c.p.m./µg. Probes for S1 nuclease analysis were synthesized by 3' end labelling of appropriate restriction fragments using the Klenow fragment of DNA polymerase I in a 'fill-in' reaction with [ $\alpha$ - $^{32}\text{P}$ ]dNTPs or by 5' end labelling using T4 polynucleotide kinase and [ $\gamma$ - $^{32}\text{P}$ ]ATP. Specific activities obtained were approx.  $5 \times 10^5$  c.p.m./µg.

**Hybridizations and S1 nuclease analysis.** Hybridization conditions for S1 mapping were according to the method of Favaloro *et al.* (1980). In most cases 20000 c.p.m. of end-labelled probe were ethanol-precipitated with 15 µg of total RNA from visna virus-infected WSCP cells. Samples were redissolved in 30 µl of hybridization buffer (80% formamide, 0.4 M-NaCl, 1 mM-EDTA, 10 mM-PIPES pH 6.4) and denatured by heating to 85 °C for 10 min, prior to hybridization for 16 h at 49 °C, or 39 °C for shorter probes. Fifteen microlitres of sample was then diluted into each of two 300 µl volumes of S1 digestion buffer (0.28 M-NaCl, 0.05 M-sodium acetate pH 5.0, 4.5 mM-ZnSO<sub>4</sub>, 10 µg/ml denatured sheared calf thymus DNA, 10 µg/ml native sheared calf thymus DNA). One of the aliquots contained S1 nuclease (1000 units except where stated). Both samples were incubated for 30 min at 30 °C. Digestions were stopped by adding 100 µl of 25 mM-EDTA, 50 mM-Tris-HCl pH 8.0, containing 10 µg yeast RNA. Samples were extracted with phenol-chloroform, precipitated with ethanol and analysed by electrophoresis on polyacrylamide gels containing 8.3 M-urea.

#### RESULTS

##### *Northern blot analysis of visna virus transcription*

Infection of choroid plexus cells by visna virus (strain 1514) at an m.o.i. of approx. 0.1 produces c.p.e. beginning at 72 h post-infection (p.i.). At this time, the cells were lysed and total

1987). At least three of eliciting antibody for these. es knowledge of how infections in tissue t infection *in vivo*. In transcripts has been visna virus produces, oducts, three spliced doubly spliced. The some acceptor sites of tend these reports by econd intron, and to rmed the utilization e of a second intron, of a polyprotein from

RNA preparations were made. Analysis of viral transcripts by Northern blotting, using a series of subgenomic viral probes (Fig. 1), showed that the virus produces a complex series of transcripts, including a full-length genomic transcript and five major spliced transcripts; the latter form two related groups. The first group comprising the three largest (4.9 kb, 4.3 kb and 4.0 kb) do not hybridize to *gag* and *pol* gene sequences (probes 1 to 3), but do hybridize to *env* and regions 3' of *env*, suggesting that they contain only a single intron. In addition, the 4.9 kb and 4.3 kb species hybridize to probes extending 5' to *env* and into the S ORF (Sonigo *et al.*, 1985). Although there is hybridization to a number of subgenomic species in the region of ORF Q, the hybridization pattern is less well defined than with the other probes and no predominant species are seen.

The two smaller RNAs (1.7 and 1.4 kb) showed a more complex pattern, hybridizing to sequences in the middle of the genome and at each end, but not to *gag*, *pol* or the central regions of the *env* gene. Initial analysis of these transcripts revealed probes specific for the S reading frame (probe 5) and a probe which overlapped S and *env* (probe 6); only the latter hybridized to the 1.4 kb RNA. The 1.7 kb RNA could therefore contain the short ORF S, whereas the 1.4 kb RNA could contain the short ORF T (Vigne *et al.*, 1987) and sequences in the 5' region of *env*. In addition both transcripts hybridize to probes 9 and 10, and could contain the F ORF. No significant differences were seen when polyadenylated RNA was used instead of total RNA (data not shown). The transcripts detected are summarized in Fig. 8. These data were in close agreement with the published studies of Davis *et al.* (1987) and of Vigne *et al.* (1987).

#### *Splicing pattern of the virus: S1 mapping*

To map the coding capacity of the spliced transcripts fully, the intron/exon boundaries of transcripts were determined by S1 mapping.

#### *Donor sites of the second intron*

Two different end-labelled probes were used to determine donor sites in the region of the 5' end of *env*. S1 nuclease protection by infected cell RNA revealed a major donor site at the apparent nucleotide (nt) position of 5982  $\pm$  2 (using the numbering of Sonigo *et al.*, 1985) (Fig. 2a, c). This is close to a consensus splice donor sequence (Mount, 1982) at nt 5971. The likely reason for the discrepancy between the apparent position of this site and the position of the consensus element is seen by considering the sequence of the second major intron acceptor site. The sequence of the spliced transcript following this acceptor matched that of the probe at seven of 11 positions after the putative donor site, and therefore may have afforded protection across these extra residues. The protected fragment seen in this and all the subsequent analyses reported here was found only when infected cell RNA was used, and not with mock-infected cell RNA. When the probe used was labelled at nt position 5480, this was the only protection seen, whereas with the probe labelled at the *Hpa*II site at nt 5904 a second protected band was seen, corresponding to an end of protection at nt 6097 (Fig. 2b). Although this signal is weaker, it is seen easily if the hybridization temperature is raised to prevent stable hybridization of the short region nt 5904 to nt 5971. Again a good fit to consensus donor sequences is found at nt 6097. In addition several minor protections are seen upstream of nt 6150. Consensus splice donor sites at nt 6159 and 6198 may give rise to minor splicing variants.

#### *Major acceptor site of the second intron*

Probes to map the major acceptor site at the end of the second intron were produced by labelling the *Hind*III site at nt position 8643 using T4 polynucleotide kinase. Using the same RNA preparations, a major protected species of about 123 bases is found (Fig. 3). A consensus acceptor element at nt 8529 is in good agreement with the likely splice junction. The use of this splice acceptor in connection with the donor junction mapped at nt 5971 would lead to the utilization of a long ORF prior to, but in frame with, the methionine codon at nt 8691, which is the first methionine encoded within ORF F (Sonigo *et al.*, 1985). This reading frame would be initiated by a methionine codon upstream of the second intron (that also used by *env*) and has some sequence similarity to the *rev* gene of HIV-1 (see Fig. 4). The use of the splice donor site at

ells (Weybridge cell line,  $\times$  multiplicity were used. re harvested for use as a approx. 0.1 was used (as arified by centrifugation it 37 °C. The monolayers 1 DMEM containing 1% sed as a source of RNA. um thiocyanate method A were fractionated by 20 mM-sodium phosphate argan *et al.*, 1986). a virus strain 1514 was a of restriction fragments er figures). Some of the used directly to produce ed labelling (Feinberg & lysis were synthesized by polymerase I in a 'fill-in' and [ $\gamma$ -32P]ATP. Specific

according to the method precipitated with 15  $\mu$ g of hybridization buffer (80% to 85 °C for 10 min, prior ple was then diluted into : pH 5.0, 4.5 mM-ZnSO<sub>4</sub>, NA). One of the aliquots ed for 30 min at 30 °C. containing 10  $\mu$ g yeast alysed by electrophoresis

n.o.i. of approx. 0.1 s were lysed and total

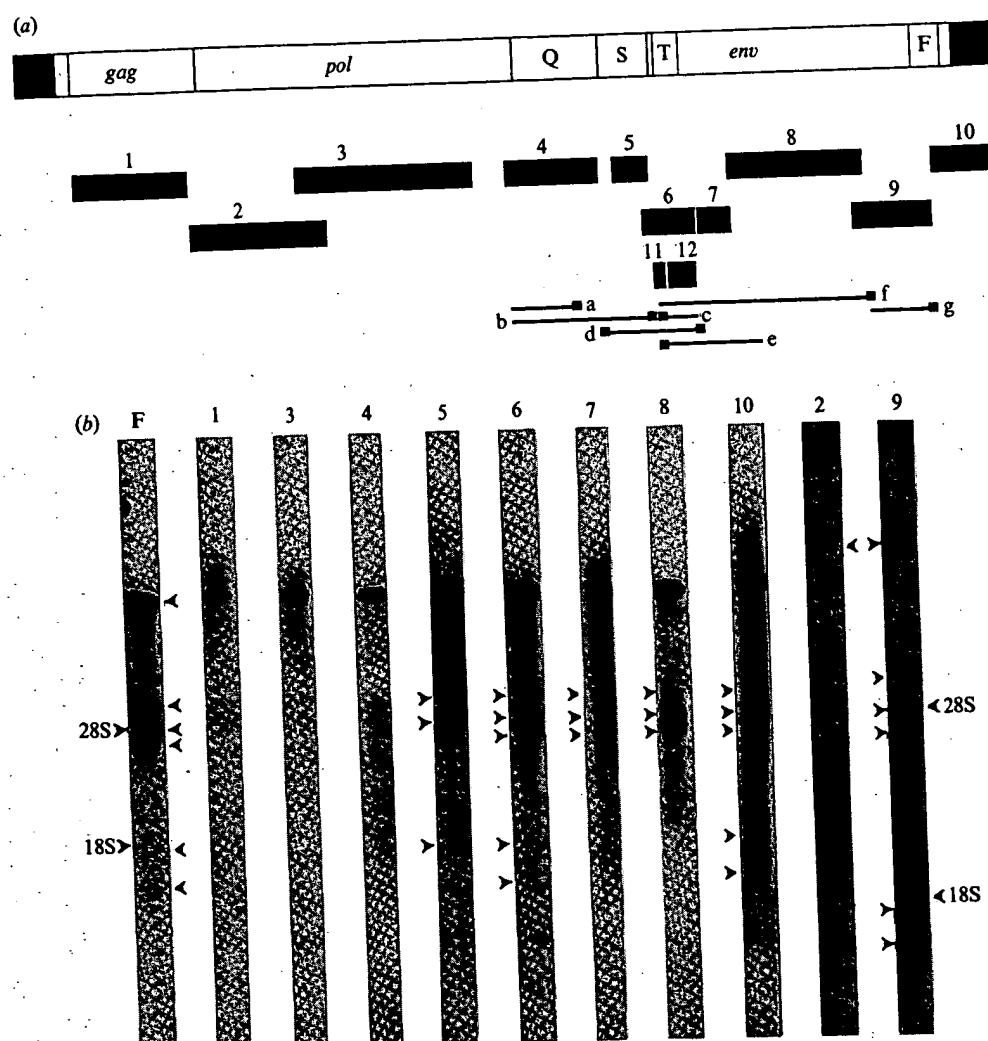


Fig. 1.(a) The genome structure of visna virus, and the probes used in Northern blot hybridizations (numbered) and in S1 mapping (designated by letters). Black boxes at each end of the genome represent the long terminal repeats. The open reading frames shown are given the designations of Sonigo *et al.* (1985). Probes 2, 5 and 7 were derived by subcloning and the remaining clones by restriction digestion. The probes were: probe 1 (nucleotides 532 to 1597); 2 (1597 to 2908); 3 (2587 to 4261); 4 (4587 to 5480); 5 (5640 to 5904); 6 (5869 to 6394); 7 (6394 to 6678); 8 (6678 to 7767); 9 (6671 to 8643); 10 (8643 to 9202); 11 (5904 to 6008); 12 (6008 to 6394); a (4597 to 5216); b (4597 to 5904); c (5904 to 6394); d (5480 to 6394); e (5904 to 6882); f (5967 to 7974); g (5974 to 8643); numbering according to Sonigo *et al.* (1985). The labelled end of the S1 probes is shown (probe d was labelled at either end in separate experiments). (b) Northern blot of total RNA from visna virus 1514-infected WSCP cells, using the probes described above as shown. F, full-length probe. (Strips hybridized against probes 2 and 9 are from a separate experiment.) Arrowheads represent the full-length visna virus transcript and major spliced products.

nt 6097 in combination with this site is not likely to allow the production of any protein product from the pre-F ORF, because the methionine that opens ORF T is not in register with this ORF. It is notable that immediately before the methionine codon at nt 8691 (amino acid position 60 in Fig. 4) there is a sequence similar to the consensus sequence for retroviral protease substrates noted by Pear & Taylor (1987). It is possible that in visna virus, F protein and a *rev*-like protein are produced as a polyprotein and subsequently cleaved.

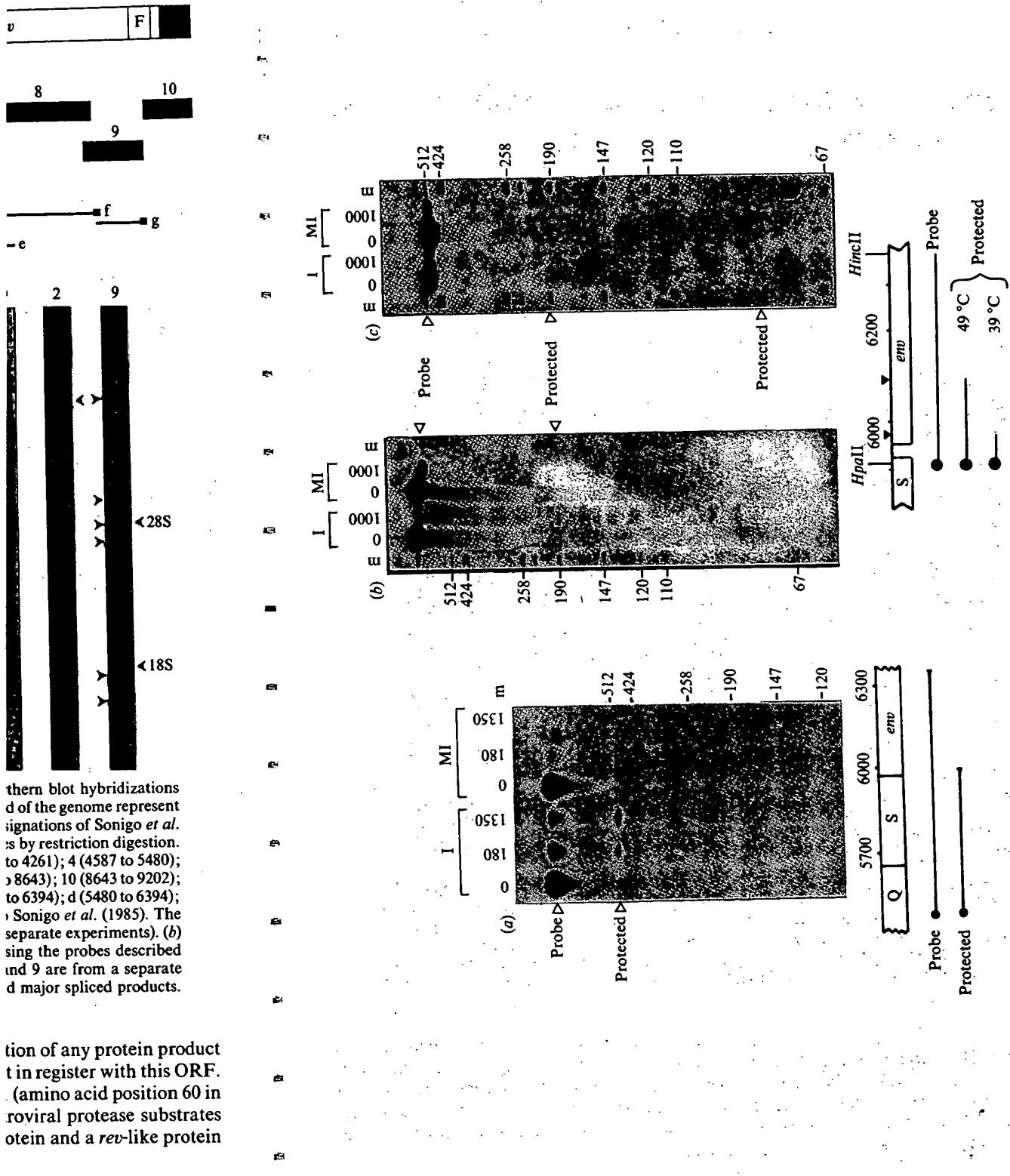


Fig. 2. S1 nuclease analysis of visna virus transcripts in RNA from infected WSCP cells. Donor sites of the second exon. (a) Probe d (nt 5480 to 6394) was labelled at position nt 5480 by a fill-in reaction. Marker sizes are shown. The schematic diagram illustrates an apparent protection ending at nt 5982. (b) Probe e (nt 5904 to 6882) was labelled at nt 5904 by a fill-in reaction. Hybridization temperature was 49 °C. (c) Probe c (nt 5904 to 6394) was labelled at nt 5904 by a fill-in reaction. Hybridization temperature was 39 °C. Major apparent protections were at nt 6097 (b) and nt 5971 (c). Marker sizes (bases) are shown (m), and S1 concentrations (units) are indicated above the lanes. RNAs from visna virus-infected (I) or mock-infected (MI) cell samples are arranged in lanes as shown.

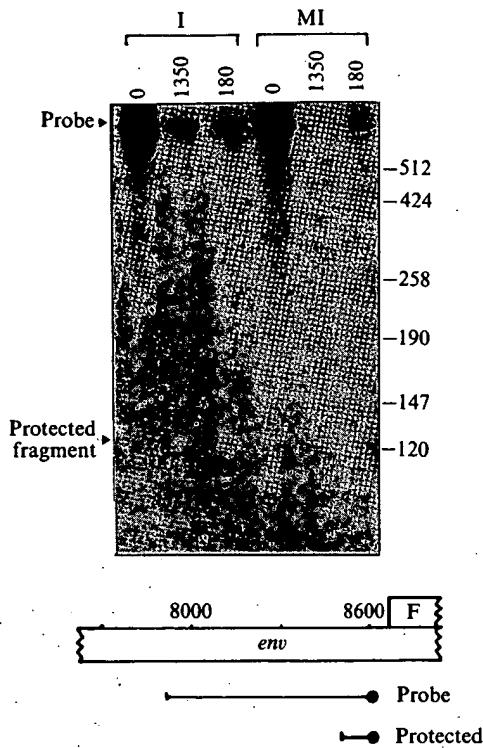


Fig. 3. S1 nuclease mapping. Major acceptor site of the second intron. Probe g (nt 7974 to 8643) was labelled at nt 8643 using polynucleotide kinase. The major apparent protection ends at nt 8529. Marker sizes are shown. RNAs from visna-infected (I) and mock-infected (MI) cells and S1 concentrations are indicated as in Fig. 2.

#### Acceptor sites of the first intron

We looked for acceptor sites 5' to the S and *env* ORFs using appropriate probes (Fig. 5). Fragments strongly protected by infected cell RNA from S1 nuclease had sizes corresponding to acceptor sites at nt positions 5590, 5954 and 6019. The first of these was barely visible when a probe labelled at nt 6394 was used (Fig. 5a, solid arrow on figure), but was more prominent with a probe labelled at nt 5904 (Fig. 5b), suggesting that many processed transcripts using the nt 5590 acceptor site are often further spliced to remove sequences including nt 6394. These positions were in good agreement with consensus acceptor sequences, and were in suitable positions to correspond to the spliced *env* containing messages of 4.3 and 4.0 kb respectively. The first site might also be used by doubly spliced messages encoding S and the pre-F transcripts and the second could encode pre-F alone. It is notable that the third site, when used in conjunction with the donor site at nt 6097, closely brackets the short T ORF.

We further looked for acceptor sites 5' to ORF Q (Fig. 5c). In agreement with Davis *et al.* (1987), we found several protected fragment sizes in this region, with no outstandingly strong protections. Of the many protected fragments seen, the strongest corresponded to putative splice acceptors at nt positions 4726, 4776, 4846 and 5006. All of these positions are close to consensus splice acceptor sequences (nt 4728, 4778, 4842 and 4992), though that at nt 4992 is not preceded by a pyrimidine-rich sequence. One of these sites (at nt 4842) has also been identified by Vigne *et al.* (1987). In that report, the short probe used makes it unlikely that the surrounding sites would have been seen. In Northern blotting, no distinct bands had been seen when ORF Q-containing sequences were used as a probe. We therefore believe that there is no strong preference in the selection of the splice acceptor in transcripts encoding Q, but that all possible consensus elements are used.

#### Other minor splice sites

A search for other protections indicative of other possible splice sites was undertaken using longer end-labelled probes. This revealed two minor protections, corresponding to splice

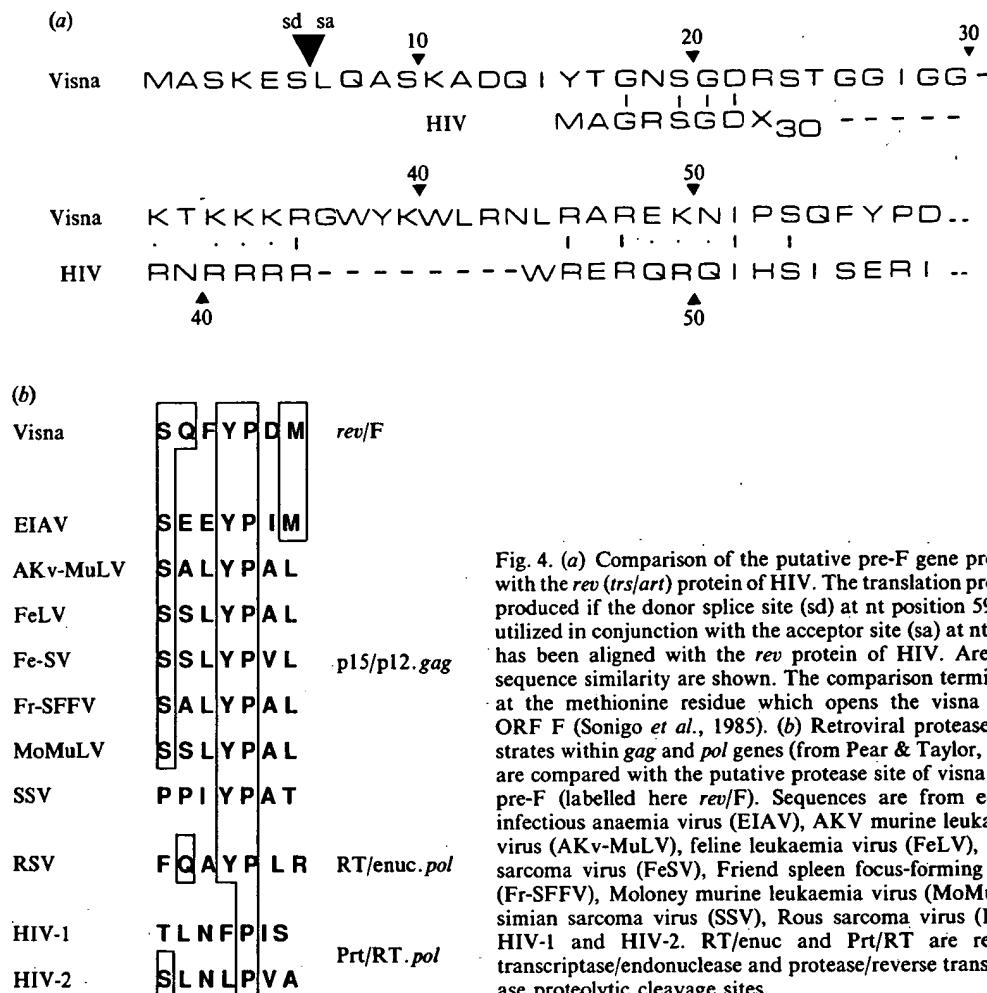


Fig. 4. (a) Comparison of the putative pre-F gene product with the *rev* (*trs/art*) protein of HIV. The translation product produced if the donor splice site (sd) at nt position 5971 is utilized in conjunction with the acceptor site (sa) at nt 8521 has been aligned with the *rev* protein of HIV. Areas of sequence similarity are shown. The comparison terminates at the methionine residue which opens the visna virus ORF F (Sonigo *et al.*, 1985). (b) Retroviral protease substrates within *gag* and *pol* genes (from Pear & Taylor, 1987) are compared with the putative protease site of visna virus pre-F (labelled here *rev/F*). Sequences are from equine infectious anaemia virus (EIAV), AKV murine leukaemia virus (AKv-MuLV), feline leukaemia virus (FeLV), feline sarcoma virus (Fe-SV), Friend spleen focus-forming virus (Fr-SFFV), Moloney murine leukaemia virus (MoMuLV), simian sarcoma virus (SSV), Rous sarcoma virus (RSV), HIV-1 and HIV-2. RT/enuc and Prt/RT are reverse transcriptase/endonuclease and protease/reverse transcriptase proteolytic cleavage sites.

e mapping. Major acceptor site in. Probe g (nt 7974 to 8643) was 3 using polynucleotide kinase. Protection ends at nt 8529. shown. RNAs from visna-  
cock-infected (MI) cells and S1 indicated as in Fig. 2.

appropriate probes (Fig. 5). These had sizes corresponding to those was barely visible when a but was more prominent with passed transcripts using the nt 6394. These sequences, and were in suitable 3 and 4.0 kb respectively. The and the pre-F transcripts and, when used in conjunction agreement with Davis *et al.* with no outstandingly strong responded to putative splice sites are close to consensus that at nt 4992 is not preceded so been identified by Vigne *et al.* the surrounding sites would been when ORF Q-containing is no strong preference in the that all possible consensus

e sites was undertaken using ns, corresponding to splice

acceptor sites at positions close to nt 7250 and 7660 (Fig. 6). Consensus acceptor sites exist at nt 7249 and 7655, but the significance of the use of these sites is not clear, as neither is followed by any long ORF (other than that of *env*).

#### Assignment of splice sites for the 1.4 and 1.7 kb mRNAs

To elucidate further the splicing pattern of 1.7 and 1.4 kb transcripts close to the start of the *env* gene, probes spanning the region nt 5904 to 6008 and nt 6008 to 6394 were prepared. Both probes hybridized to the 1.7 kb transcript (Fig. 7), suggesting that it is formed through splicing at the donor site at nt 6097 (although some transcripts may use instead the site at nt 5971). Hybridization of the 1.4 kb transcript to the nt 5904 to 6008 probe could not be detected although the probe further downstream does hybridize. This suggests that the majority of these transcripts also use the donor site at nt 6097. It is unlikely that transcripts containing only sequences from nt 5954 to 5971 would be detected in our blots, but it is clear that the majority of the 1.4 kb transcript does not contain second exon sequences 5' to nt 5954. It must therefore use the acceptor sites at nt 5954 or 6019. The RNA preparation used in this experiment contained lower concentrations of *env*-containing messages than that used in Fig. 1. We have subsequently found that this is characteristic of earlier stages of infection (unpublished data).

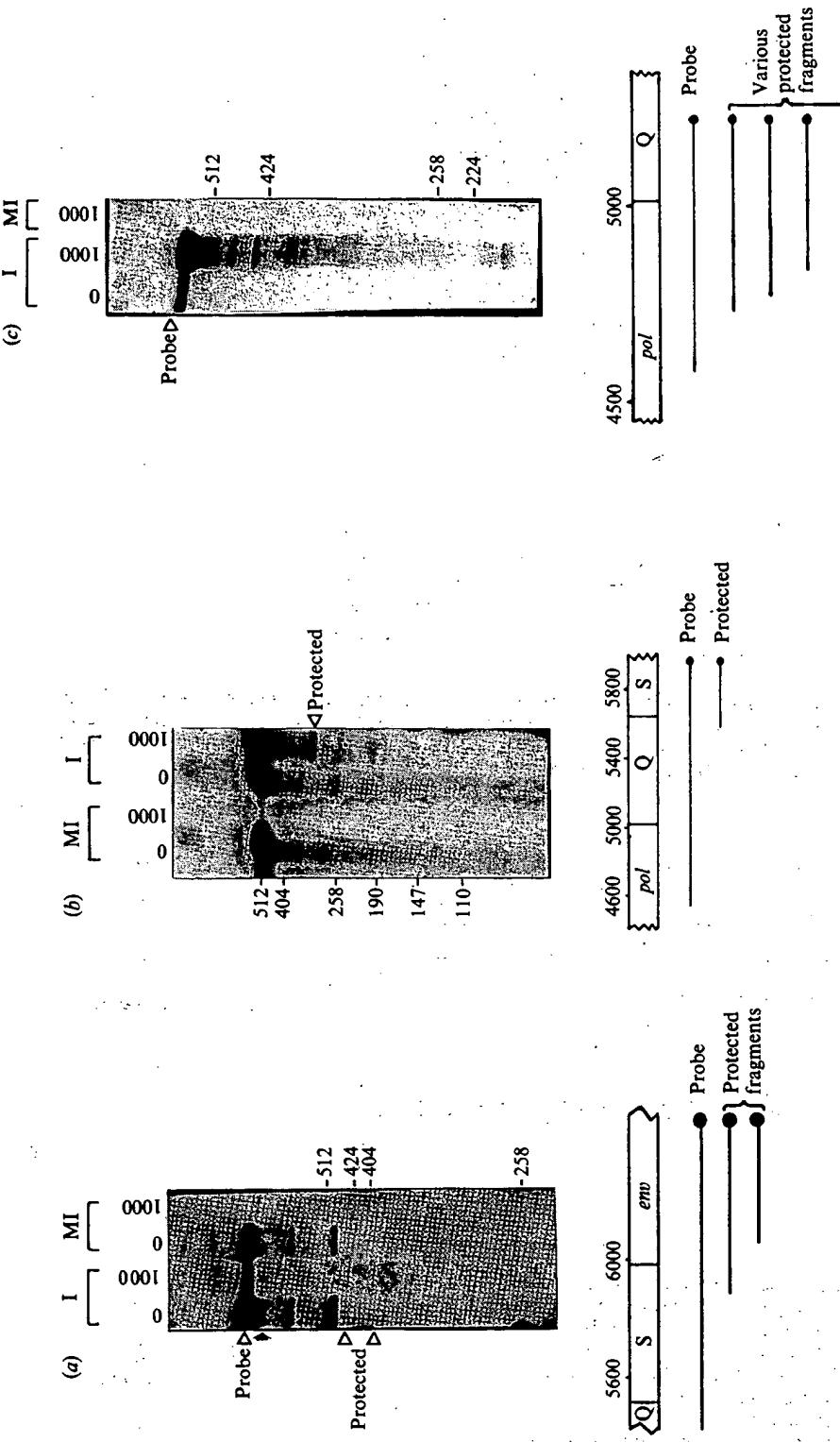


Fig. 5. S1 nuclease mapping data showing acceptor sites of the first intron. (a) Sites close to the 5' end of *env*. Probe d (nt 5480 to 6394) was labelled at the nt 6394 site using T4 polynucleotide kinase. The two additional bands in the probe are pBR322-specific. The solid arrow shows a minor protected species. Major protected species end at nt 5954 and 6019. (b) Sites close to the 5' end of ORF S. Probe b (nt 4597 to 5904) was labelled at position 5904 using polynucleotide kinase. The curved upper band on this gel is the result of a compression artefact. The major protected species ends at nt 5590. (c) Sites close to the 5' end of ORF Q. Probe a (nt 4597 to 5216) was labelled at the nt 5216 site using polynucleotide kinase. RNAs from visna-infected (I) and mock-infected (MI) cells are in lanes indicated in the figure. Marker sizes are shown.

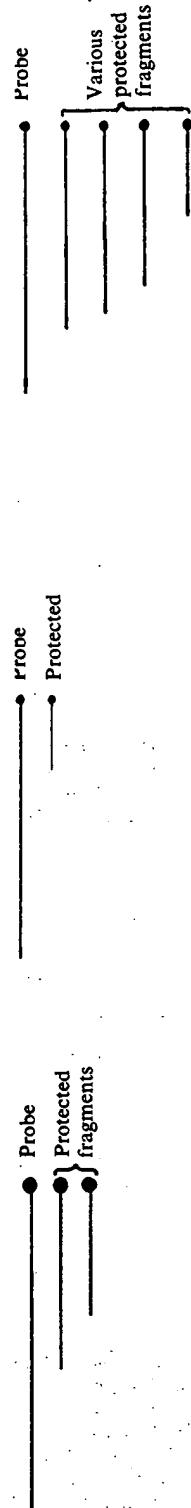


Fig. 5. S1 nuclease mapping data showing acceptor sites of the first intron. (a) Sites close to the 5' end of *env*. Probe d (nt 5480 to 6394) was labelled at the nt 6394 site using T4 polynucleotide kinase. The two additional bands in the probe are pBR322-specific. The solid arrow shows a minor protected species. Major protections end at nt 5954 and 6019. (b) Sites close to the 5' end of ORF S. Probe b (nt 4597 to 5904) was labelled at position 5904 using polynucleotide kinase. The curved upper band on this gel is the result of a compression artefact. The major protected species ends at nt 5590. (c) Sites close to the 5' end of ORF Q. Probe a (nt 4597 to 5216) was labelled at the nt 5216 site using polynucleotide kinase. RNAs from visna-infected (I) and mock-infected (MI) cells are in lanes indicated in the figure. Marker sizes are shown in lanes indicated in the figure. Marker sizes are indicated in the figure.

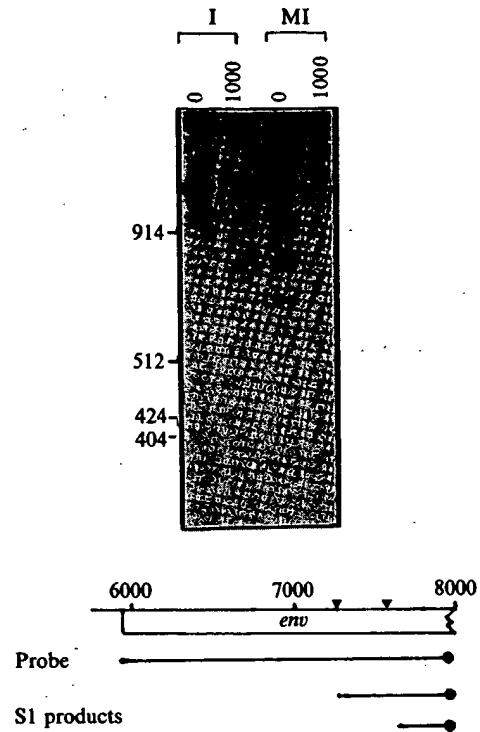


Fig. 6. S1 nuclease mapping data showing minor acceptor sites in the second intron. Probe f (nt 5967 to 7974) was labelled at position 7974 with T4 polynucleotide kinase. The protected species seen end at nt positions 7259 and 7655. RNAs from visna-infected (I) or mock-infected (MI) cells are in lanes indicated in the figure. Marker sizes are shown on the left.

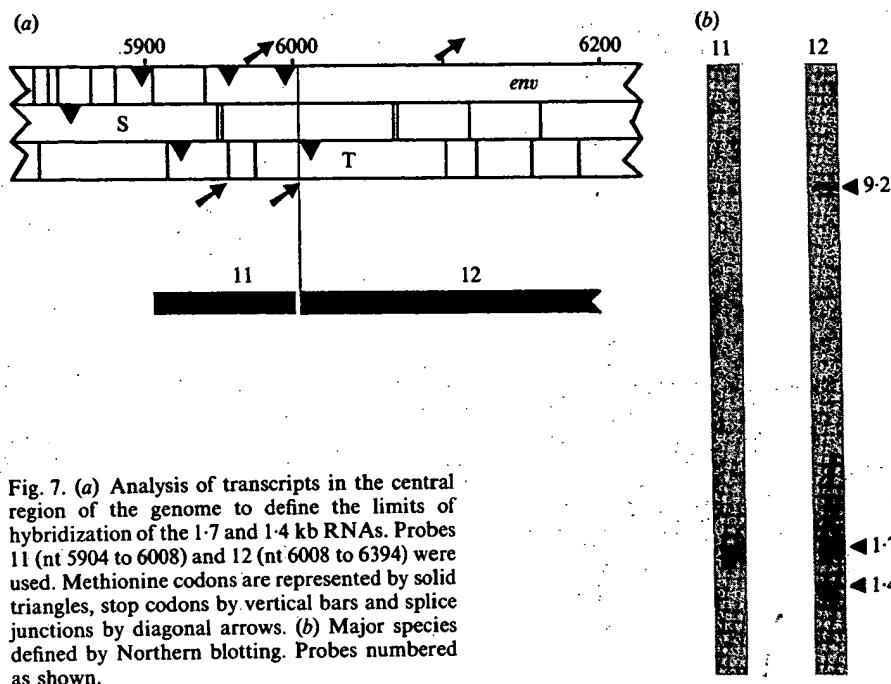


Fig. 7. (a) Analysis of transcripts in the central region of the genome to define the limits of hybridization of the 1.7 and 1.4 kb RNAs. Probes 11 (nt 5904 to 6008) and 12 (nt 6008 to 6394) were used. Methionine codons are represented by solid triangles, stop codons by vertical bars and splice junctions by diagonal arrows. (b) Major species defined by Northern blotting. Probes numbered as shown.

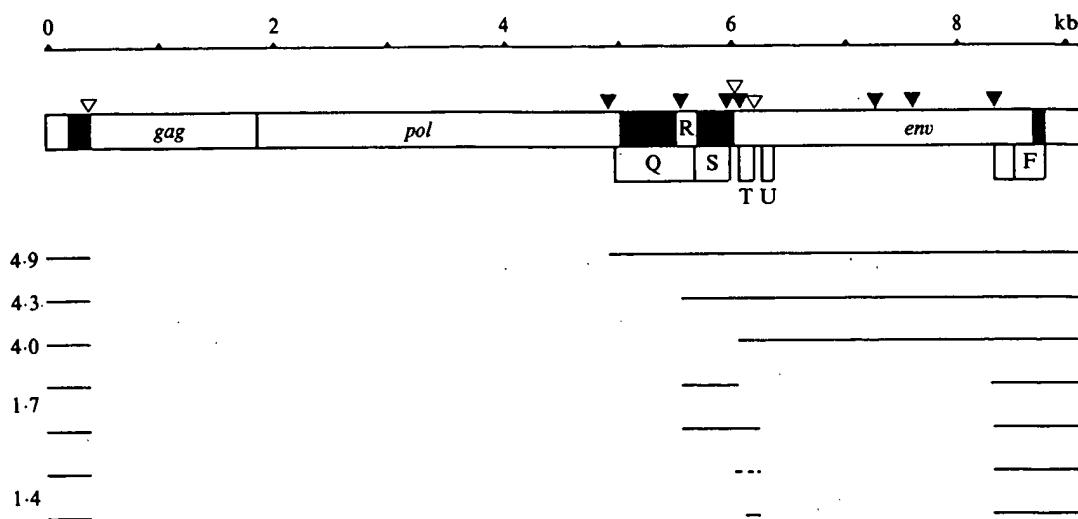


Fig. 8. Map of the major transcripts. The RNAs are shown as lines, splice donor sites as open triangles and splice acceptor sites as solid triangles.

#### DISCUSSION

The genome structure of visna-maedi virus is similar to that of other members of the lentivirus group in that there are a number of small ORFs that may be utilized to produce small proteins with regulatory or other functions (Sonigo *et al.*, 1985; Braun *et al.*, 1987). The virus produces a diffusible factor capable of trans-activation of its autologous promoter (Hess *et al.*, 1985). Sequence homologies with the *tat* protein of HIV suggest that this factor may be encoded by ORF S. In addition a second short ORF of HIV, known as *rev*, *trs* or *art*, controls splicing or export from the nucleus of transcripts in such a way that the ratio of spliced to unspliced transcripts is decreased in the absence of this protein (Feinberg *et al.*, 1986; Sadaie *et al.*, 1988). No ORF homologous to *trs* has previously been reported in visna virus. However it has been reported that spliced transcripts are produced early during visna virus infection, whereas unspliced transcripts are present only at later times (Vigne *et al.*, 1987; D. R. Sargan & R. G. Dalziel, unpublished results) although Davis *et al.* (1987) present conflicting results.

The transcripts produced by visna virus in choroid plexus cells exhibit a complex splicing pattern, in which either no introns or one or two may be removed. From the data presented here and summarized in Fig. 8, it is clear that ORF S may be contained in the doubly spliced transcript of 1.7 kb, using a splice acceptor site at nt position 5590; such a transcript would include a termination codon in the first coding exon, and so would be unlikely to utilize ORFs in the last exon. The other abundant doubly spliced RNA includes a short central exon attached to an ORF encoding a pre-F protein. The first 51 amino acids of the final exon of this ORF show limited similarity to the *rev* protein of HIV (particularly in two groups of very basic residues after nt positions 25 and 44 of this exon). They are immediately followed by a sequence with homology to known proteolytic cleavage sites of retroviral protease substrates, followed by the sequences previously referred to as ORF F.

Other authors have reported the sequences used by visna virus as the donor splicing site and the extreme 5' acceptor sites of the first intron. Here we have looked for other major splice junctions used by the virus. We cannot rule out the possibility that we have overlooked some junctions used in this infection type and it is also possible that a different pattern of transcripts is found *in vivo*. It is not clear how the choice of splice junction elements used by the virus occurs. All the splice sites found show reasonable fits to published consensus sequences (Fig. 9; Mount,

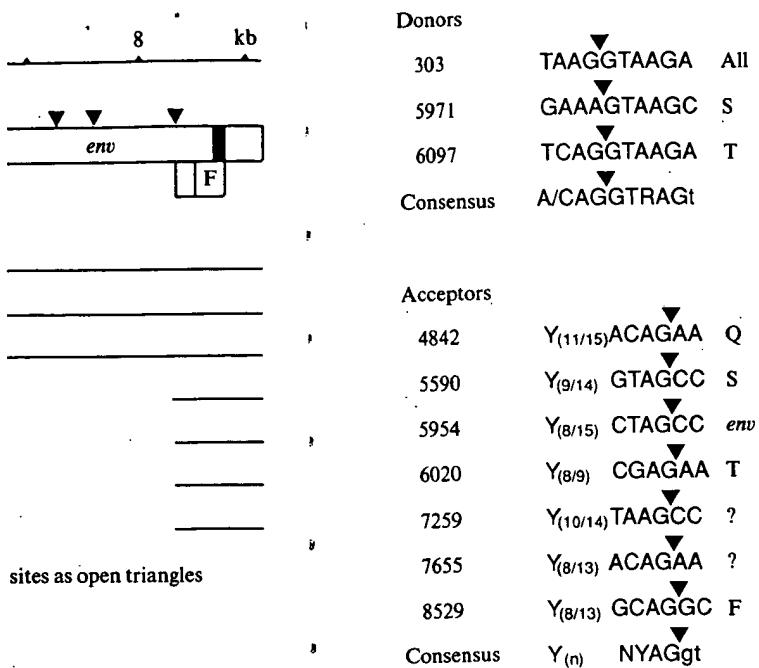


Fig. 9. Comparison of the sequences of splicing sites reported in this paper with consensus splice sites reported by Mount (1982). The donor site at position 303 and the acceptor site at position 4842 were reported by others (Davis *et al.*, 1987; Vigne *et al.*, 1987).

other members of the utilized to produce small *et al.*, 1987). The virus promoter (Hess *et al.*, factor may be encoded *art*, controls splicing or spliced to unspliced 36; Sadaie *et al.*, 1988). However it has been virus infection, whereas D. R. Sargan & R. G. Sargan, unpublished results.

bit a complex splicing the data presented here in the doubly spliced such a transcript would likely to utilize ORFs in central exon attached to exon of this ORF show very basic residues after sequence with homology owed by the sequences

donor splicing site and for other major splice have overlooked some pattern of transcripts is sed by the virus occurs. uences (Fig. 9; Mount,

1982). Preliminary experiments to measure the size of exons used in the central regions of the visna virus genome have suggested that either of the last two acceptor sites of the first intron may be used with the second exon donor site at nt 6097. However, transcripts in which the acceptor site used is upstream of nt 5480 use the donor site at nt 5971 preferentially (Fig. 2a and data not shown). Previous reports and our own unpublished observations suggest that the proportions of spliced and unspliced transcripts vary with time after infection in lytic visna virus infections. In HIV, the *rev* gene product acts in the nucleus and has a role in regulating the level of spliced transcripts found in the cytoplasm (Cullen *et al.*, 1988; Sadaie *et al.*, 1988). The control of the concentration of spliced transcripts in visna virus is not at the level of RNA stability of spliced versus unspliced transcripts (D. R. Sargan, unpublished results), and therefore may occur by a mechanism similar to that utilized by HIV.

*Note added in proof.* After this work was completed, Mazarin *et al.* (1988; *Journal of Virology* 62, 4813-4818) reported a visna virus cDNA clone encoding a *rev*-like protein which they have termed *vpe1*.

We would like to thank Drs J. E. Clements and O. Narayan for the visna virus 1514 DNA clone used as a basis for these studies. We also thank many colleagues for helpful discussions. The work was supported by a Project Development Award from the Wellcome Trust.

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## A New-Generation Stable Inducible Packaging Cell Line for Lentiviral Vectors

DEBORAH FARSON,<sup>1</sup> ROCHELLE WITT,<sup>1</sup> RYAN McGUINNESS,<sup>1</sup> TOM DULL,<sup>1</sup> MICHAEL KELLY,<sup>1</sup> JINPING SONG,<sup>1</sup> ROBERT RADEKE,<sup>1</sup> ANATOLY BUKOVSKY,<sup>1</sup> ANTONELLA CONSIGLIO,<sup>2</sup> and LUIGI NALDINI<sup>3</sup>

### ABSTRACT

We have successfully generated and characterized a stable packaging cell line for HIV-1-based vectors. To allow safe production of vector, a minimal packaging construct carrying only the coding sequences of the HIV-1 *gag-pol*, *tat*, and *rev* genes was stably introduced into 293G cells under the control of a Tet<sup>o</sup> minimal promoter. 293G cells express the chimeric Tet<sup>R</sup>/VP16 *trans*-activator and contain a tetracycline-regulated vesicular stomatitis virus protein G (VSV-G) envelope gene. When the cells were grown in the presence of tetracycline the expression of both HIV-1-derived and VSV-derived packaging functions was suppressed. On induction, approximately 50 ng/ml/24 hr of Gag p24 equivalent of vector was obtained. After introduction of the transfer vector by serial infection, vector could be collected for several days with a transduction efficiency similar or superior to that of vector produced by transient transfection both for dividing and growth-arrested cells. The vector could be effectively concentrated to titers reaching 10<sup>9</sup> transducing units/ml and allowed for efficient delivery and stable expression of a GFP transgene in the mouse brain. The packaging cell line and all vector producer clones described here were shown to be free from replication-competent recombinants, and from recombinants between packaging and vector constructs that transfer the viral *gag-pol* genes. The packaging cell line and the assays developed will advance lentiviral vectors toward the stringent requirements of clinical applications.

### OVERVIEW SUMMARY

The production of lentiviral vectors poses significant challenges due to the hybrid nature of the vectors, the complexity of the lentiviral genome, and the toxicity of several components of the vector particle. Accordingly, lentiviral vectors have been produced until now by transient cotransfection of the required constructs, an approach hardly amenable to the stringent characterization and scale-up required for clinical applications. Here we report the successful development and characterization of a stable inducible packaging cell line for HIV-1-derived lentiviral vectors. To allow safe and efficient production of vector we used a minimal set of HIV-derived genetic information and a tetracycline-dependent system to control expression of packaging and envelope functions. We selected clones with

undetectable basal expression and efficient release of vector particles on induction. By serial infection of the transfer construct, producer clones could be obtained that yielded vectors with titer, infectivity, and performance matching those of vector obtained by transient transfection. In addition, these vectors allowed for efficient delivery and stable transgene expression in the mouse brain. We also developed sensitive assays for replication-competent recombinants and replication-defective recombinants transferring viral packaging functions. The packaging cell line described here and all vector producer clones derived from it were shown to be free from either type of recombinant. The scalable and safe vector production allowed by this new packaging cell line, and the assays developed for its characterization, should advance the applications of lentiviral vectors for gene therapy.

<sup>1</sup>Cell Genesys, Foster City, CA 94404.

<sup>2</sup>Telethon Institute for Gene Therapy, H.S. Raffaele, 20132 Milan, Italy.

<sup>3</sup>Institute for Cancer Research and Treatment, University of Torino Medical School, 10060 Candiolo, Italy.

## INTRODUCTION

**L**ENTIVIRAL VECTORS are replication-defective, hybrid viral particles consisting of the core proteins and enzymes of a lentivirus and the envelope of a different virus. They transduce several types of cells and tissues independent of cell division and allow long-term expression of the transgene after direct injection into adult rodents (Naldini *et al.*, 1996a; Naldini, 1998).

Compared with conventional murine leukemia virus (MuLV)-based retroviral vectors, the production of lentiviral vectors poses significant challenges due to the hybrid nature of the vectors, the complexity of the lentiviral genome, and the toxicity of several components of the vector particle.

The hybrid nature of the vectors dictates the use of three different plasmids to produce infectious particles (Naldini, 1998). Two of the plasmids encode the proteins that assemble the particle, one plasmid expressing the core proteins and enzymes of human immunodeficiency virus type 1 (HIV-1) (packaging construct), and the other expressing the envelope protein of an unrelated virus, most often the protein G of the vesicular stomatitis virus (VSV-G). The third construct contains an expression cassette for the transgene flanked by HIV-1 *cis*-acting sequences required for encapsidation (McBride and Panganiban, 1996; McBride *et al.*, 1997), reverse transcription, and integration (transfer vector construct).

The complexity of lentiviral genomes is due to the presence, in addition to the structural *gag*, *pol*, and *env* genes common to all retroviruses, of two regulatory genes, *tat* and *rev*, whose products are essential for expression of the genome in the context of its native state, and a set of accessory genes that are not essential for viral replication but are critical for pathogenesis (Cullen, 1998). The Tat and Rev proteins act at the transcriptional and posttranscriptional level, respectively. HIV-1 Tat binds to a stem-loop structure (the *trans*-activation response [TAR] element) in the nascent long terminal repeat (LTR) RNA and tethers the cyclin T-CDK9 complex to polymerase II, promoting transcriptional elongation (Wei *et al.*, 1998; Bieniasz *et al.*, 1999). HIV-1 Rev binds to an RNA motif (Rev-responsive element, RRE) found in the envelope-coding region of the transcript and bridges it to the nuclear export factor exportin 1 (CREM), promoting cytoplasmic export of unspliced and singly spliced viral transcripts that express late viral proteins (Fornerod *et al.*, 1997; Neville *et al.*, 1997; Stade *et al.*, 1997). Additional components of the Rev-dependent switch of *gag-pol* expression are suboptimal splice acceptor sites downstream of the genes that facilitate accumulation of unspliced transcripts (Staffa and Cochrane, 1994), and inhibitory sequences within the *gag-pol* coding frames that promote degradation of unspliced transcripts in the absence of active export (Schneider *et al.*, 1997).

The design of HIV-1-derived vectors reflects and exploits the complexity of the viral genome. Tat is required to activate transcription from the transfer vector LTR. Rev is required to export from the nucleus unspliced transcripts both of the transfer construct, allowing encapsidation, and of the packaging construct, allowing expression of the Gag and Pol polyproteins. As Tat and Rev are expressed *in trans* in vector-producer cells, transcription from the vector LTR, and accumulation of transcripts that can be encapsidated, are restricted in transduced

cells (Naldini *et al.*, 1996a; Naldini, 1998; Dull *et al.*, 1998). The requirement for HIV-1 accessory genes in vector production has been investigated. We previously showed that vectors packaged by a construct from which all the HIV-1 accessory genes were deleted were as efficient as vectors made from wild-type constructs at transducing genes *in vitro* and *in vivo* (Zufferey *et al.*, 1997). Similar findings have since been reported by others (Kim *et al.*, 1998; Mochizuki *et al.*, 1998; Gasmi *et al.*, 1999). As the nonessential genes of HIV-1 are critical for pathogenesis (Deacon *et al.*, 1995; Kirchhoff *et al.*, 1995; Aldrovandi and Zack, 1996), the new "minimal" packaging constructs were adopted to increase the biosafety of vector production.

Several of the proteins required to assemble lentiviral vectors are toxic to cells when overexpressed, including VSV-G (Burns *et al.*, 1993), HIV-1 Vpr (Bartz *et al.*, 1996), Tat (Li *et al.*, 1995), Rev (Miyazaki *et al.*, 1995), and protease (Konvalinka *et al.*, 1995). Accordingly, vectors are currently produced by transient cotransfection of the required constructs into human kidney 293T cells (Naldini *et al.*, 1996a). The average transient producer culture yields the vector equivalent of 100–1000 ng/ml/24 hr of Gag p24 antigen for 2–3 days, corresponding to  $10^6$ – $10^7$  transducing units (TU)/ml as measured by end-point biological titration on HeLa cells (Zufferey *et al.*, 1997; Dull *et al.*, 1998). The average particle output per cell is approximately 50–500 fg of p24/cell/24 hr and the average particle infectivity is  $10^4$  TU/ng p24 (Dull *et al.*, 1998). Purification and concentration of vector to 1000-fold higher titer are easily achieved by ultracentrifugation. Transient transfection, however, is hardly amenable to the stringent characterization, standardization, and scale-up required by good manufacturing practices of vector production for human applications. Furthermore, cotransfection may increase the risk of recombination between plasmids, thus detracting from the biosafety devices built into the vector design. The availability of a stable producer system will assist in transferring the unique advantages of lentiviral vectors to the clinic.

Various attempts at generating stable packaging cell lines for HIV-derived vectors have been reported, including the use of inducible regulatory elements to limit expression of toxic components from early generations of packaging constructs (Carroll *et al.*, 1994; Yu *et al.*, 1996; Srinivasakumar *et al.*, 1997; Kaul *et al.*, 1998; Kafri *et al.*, 1999). While these studies have been helpful in evaluating the different approaches, they have not yielded a producer with satisfactory vector output, activity, stability, or biosafety. Here we describe the successful development and detailed characterization of a new second-generation, inducible Lentikat packaging cell line for HIV-1-derived vectors. On induction approximately 100 ng/ml/24 hr of Gag p24 equivalent of vector was obtained with infectious titers above  $10^6$  TU/ml and transducing activity indistinguishable from, or superior to, that of vector produced by transient transfection. By newly developed and highly sensitive assays, we demonstrated the absence of replication-competent and replication-defective recombinants that would have transferred packaging functions in vector batches from the stable producers. This packaging cell line and the assays developed for its characterization should be of significant value.

for most applications of gene transfer mediated by lentiviral vectors.

## MATERIALS AND METHODS

### Plasmid construction and transient transfections

**Plasmids for packaging and producer cell lines.** pH2CMVGFP is a lentiviral transfer vector carrying the EGFP gene (Clontech, Palo Alto, CA) under the control of the human cytomegalovirus (CMV) immediate-early enhancer/promoter. The parental transfer vector plasmids that formed pH2CMVGFP were described previously (Dull *et al.*, 1998). pH2CMVGFP was constructed by ligating the 10.1-kb *Clal*/*Bam*HI fragment of pH2hPGK.GFP to the 827-bp *Bam*HI fragment containing the CMV enhancer/promoter of pMDLg/p. A *Clal*-to-*Bam*HI linker consisting of synthetic oligonucleotides 5'-CGATTATGG-3' and 5'-GATCCCATAAAT-3' completed the construction by spanning the fragments at the 5' end of the CMV enhancer/promoter. pCMVΔR8.75 differs from the previously described packaging plasmid pCMVΔR8.74 (Dull *et al.*, 1998) by containing an optimized Kozak sequence at the ATG of the *gag-pol* gene (Kozak, 1997). It was constructed by joining the 5.3-kb *Ngo*MI/*Sst*II and 6.6-kb *Nco*I-*Ngo*MI fragments of pCMVΔR8.74 with an *Sst*II-to-*Nco*I linker. The linker contained the HIV 5' major splice donor sequence and the Kozak consensus sequence and consisted of synthetic oligonucleotides 5'-GGGACTGGTGAATTGAGATCTGC-CGCCGC-3' and 5'-CATGGCGGGCGAGATCTGAATT-CACTCACCACTCCCAGC-3'. pTetCMVΔR8.75 was derived from pCMVΔR8.75 by swapping the CMV enhancer/promoter with the TetO7-controlled CMV promoter in the following way. The 4.6-kb *Ngo*MI-*Pst*I fragment of pCMVΔR8.74 was blunted with Klenow enzyme at the *Pst*I site and ligated to the 450-bp *Xba*I-blunted *Sst*II fragment of pTetSplice (GIBCO-BRL, Grand Island, NY) and the 6.6-kb *Sst*II/*Ngo*MI fragment of pCMVΔR8.74. The construction of the TetO7/CMV-controlled VSV-G envelope expression plasmid, pMDtet.G, was previously described (Ory *et al.*, 1996).

**Plasmids for recombinant assays.** Plasmid pR8.7ΔEnv was generated by insertion of a 4.3-kb *Bcl*II-*Xba*I fragment of plasmid pCMVΔR8.74 containing deletions of all accessory and *env* viral genes into the corresponding region of pR8 plasmid (Gallay *et al.*, 1995). To restore a full-length *env* gene in pR8.7ΔEnv, the *Sal*I-*Bam*HI fragment of pR8.7ΔEnv was replaced by a corresponding fragment of pR7 (Kim *et al.*, 1989).

**Vector generation by transient transfection.** 293T cells were plated at  $5 \times 10^6$  cells per 10-cm plate for 24 hr and refed with 10 ml of fresh medium 2 hr prior to transfection. Precipitate was formed by adding the appropriate plasmids to a final volume of 450  $\mu$ l of 0.1 $\times$  TE (10 mM Tris [pH 8.0], 1 mM EDTA [pH 8.0] diluted 1:10 with deionized H<sub>2</sub>O [dH<sub>2</sub>O]) and 50  $\mu$ l of 2.5 M CaCl<sub>2</sub>, mixing well. Five hundred microliters of 2 $\times$  HEPES-buffered saline (281 mM NaCl, 100 mM HEPES, 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.12) was then added while aerating the so-

lution with a pipette. The mixture was then added dropwise to 293T cells and swirled gently. Medium was replaced with fresh growth medium 16 hr posttransfection, and after an additional 24 hr the medium was collected, filtered through a 0.22- $\mu$ m pore size filter, and flash frozen on dry ice.

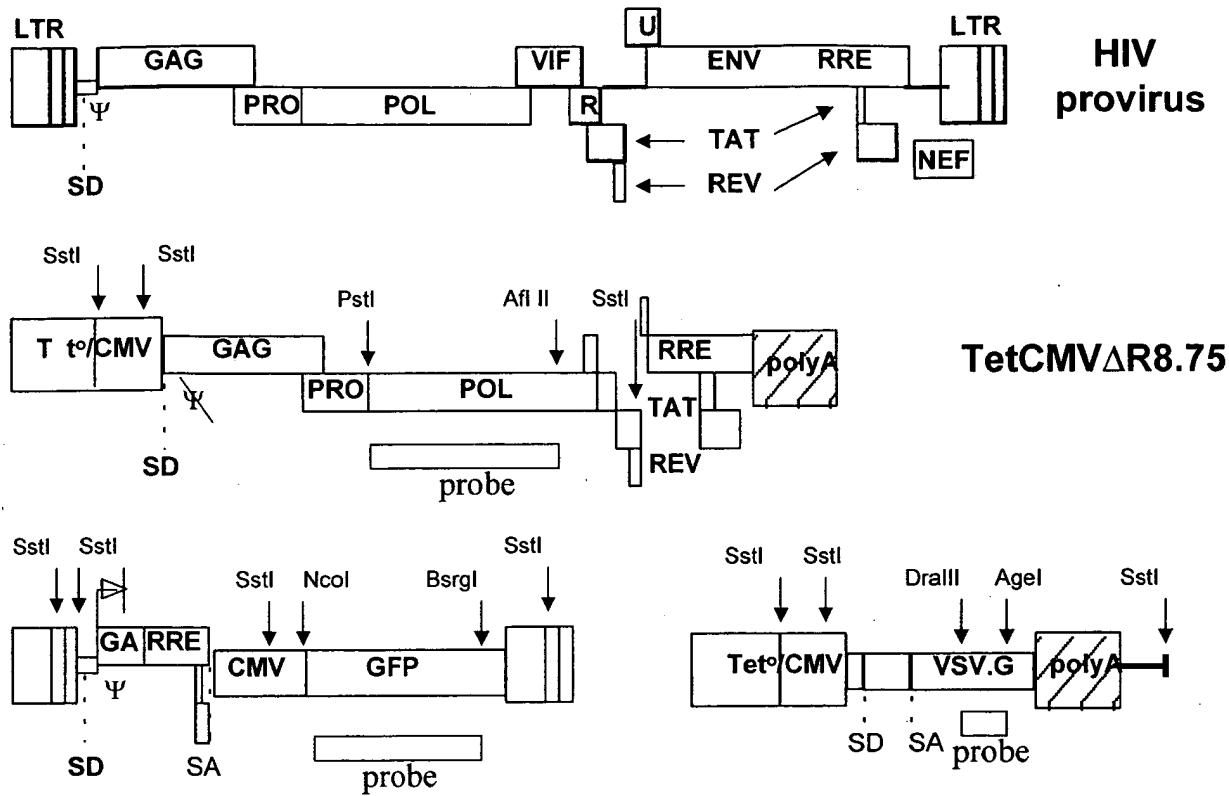
### Cells, stable packaging and producer clones, inductions, titrations

**Cells.** 293G cells (Ory *et al.*, 1996) and all 293G-derived clones were grown in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% calf serum and tetracycline (1  $\mu$ g/ml) or doxycycline (0.7  $\mu$ g/ml). 293T cells were grown in IMDM supplemented with 10% fetal bovine serum. HeLa cells (ATCC CCL-2) were grown in Dulbecco's modified Eagle's medium (DMEM)-glucose (4.0 g/liter) with 10% fetal bovine serum. All cells were split at 3 to 4-day intervals.

**Generation of Lentikat2.54.** 293G cells were plated at 0.65  $\times$  10<sup>6</sup> cells per 10-cm dish 48 hr prior to use. Medium was changed 2 hr prior to transfection. A total of 20  $\mu$ g of plasmid DNA was used per 10-cm dish: 18  $\mu$ g of the packaging plasmid pTetΔCMVΔR8.75 and 2  $\mu$ g of the zeocin resistance plasmid, pZeoSV2 (Invitrogen, Carlsbad, CA) and precipitated as described above. Medium was changed 14–16 hr posttransfection, and after an additional 24 hr the cells were split into medium supplemented with zeocin (250  $\mu$ g/ml) (Invitrogen) and tetracycline (1  $\mu$ g/ml). Colonies were picked 3–4 weeks later and characterized for p24 expression after induction in six-well plates. When cells were 90% confluent the medium was changed and after 24 hr a noninduced sample was collected, filtered through a 0.45- $\mu$ m pore size filter, and flash frozen on dry ice. The medium was replaced by induction medium, DMEM-glucose (1.0 g/liter) with 10% calf serum, and collected daily as needed. All conditioned media were filtered and frozen before assay. Conditioned media were assayed for p24 by immunocapture (Du Pont, Wilmington, DE). Packaging clones were evaluated for their ability to produce vector after two infections at 3.5–5 multiplicities of infection (MOIs) with transiently produced HR2CMVGFP vector in the presence of Polybrene (8  $\mu$ g/ml). Cells were expanded and noninduced and induced conditioned media were collected as described above. Conditioned media were assayed for p24 by immunocapture and titrated for green fluorescent protein (GFP) as described below.

**Producer clones.** Producer populations were made by the serial transduction (infections A–E) of Lentikat2.54 packaging clone with transiently produced VSV-G-pseudotyped HR2CMVGFP vector in the presence of Polybrene (8  $\mu$ g/ml). Populations were expanded, induced, and evaluated for p24 and GFP titers as described. Lentikat2.54 population E was subcloned by dilution cloning, and producer clones were induced and characterized for p24 and GFP titers as described below.

**Titer assay.** Titers were done on HeLa cells plated at 5  $\times$  10<sup>4</sup> cells per well of a 6-well plate 24 hr prior to infection. Infections were done in the presence of Polybrene (8  $\mu$ g/ml), and refed with growth medium 24 hr later. After an additional 2 to



### HR2CMVGFP Transfer Vector

### pMDtet.G

**FIG. 1.** Schematic diagrams of the HIV provirus and the three constructs used to create the Lentikat packaging and producer cell lines. The viral LTRs, the reading frames of the viral genes, the major 5' splice donor site (SD), the encapsidation signal ( $\Psi$ ), and the Rev response element (RRE) are boxed and indicated in boldface type. pTetCMVΔR8.75 expresses the *gag*, *pol*, *tat*, and *rev* genes under the control of the hybrid tetracycline operator-minimal CMV promoter (Tet<sup>o</sup>/CMV). The transfer construct HR2CMVGFP contains HIV-1 *cis*-acting sequences and an expression cassette for the transgene. pMDtet.G encodes the heterologous envelope VSV-G under control of the Tet<sup>o</sup>/CMV promoter to pseudotype the vector. Splice sites are from the  $\beta$ -globin gene. Restriction sites utilized in the Southern analysis and in generating the  $^{32}$ P-labeled probes are indicated over each diagram. Probed regions are indicated by boxes beneath each diagram. The 3' *Sst*I site shown in the diagram of pMDtet.G is in the 293 cellular genome flanking the integration site of the transfected expression cassette approximately 6.6 kb away from the internal *Sst*I site.

10 days the HeLa cells were evaluated for GFP expression by FACScan (Becton Dickinson, Mountain View, CA).

#### Southern analysis of packaging and producer cell lines

Genomic DNA (10  $\mu$ g) from each packaging or producer cell line was digested with *Sst*I and electrophoresed on a 0.7% 2-mercaptoethanol (2-ME) agarose gel, and Southern blots were transferred to Zetabind filter paper (CUNO Laboratory Products, Meriden, CT). Sequences of the *gag-pol* expression plasmid pTetCMVΔR8.75 were probed with a  $^{32}$ P-labeled, 1.9-kb *pol* fragment isolated by *Pst*I and *Afl*II digestion, and a  $^{32}$ P-labeled, 0.3-kb fragment isolated from *rev* intron 2 by *Bam*HI and *Xho*I digestion. Sequences of the envelope plasmid, pMDtet.G, were probed with a  $^{32}$ P-labeled, 990-bp fragment isolated by *Eco*RI and *Age*I digestion. Sequences of the transfer vector, HR2CMVGFP, were probed with a  $^{32}$ P-labeled, 711-bp, GFP-coding region fragment isolated from the plasmid pEGFP (Clontech) by *Nco*I and *Bsr*GI digestion. The *Sst*I-cut genomic DNAs

were also probed with a  $^{32}$ P-labeled 382-bp *Mlu*I-to-*Xba*I fragment containing coding sequences of the *ter*<sup>R/VP16 *trans*-activator from the plasmid pTet-on (Clontech). The resultant bands on the Southern blots were as follows: TetCMVΔR8.75, 4.7 kb with *Pst*I-*Afl*II probe, 6.1 kb with the *Bam*HI-*Xho*I probe; VSV-G *env*, 6.6 kb; HR2CMVGFP, 1.4 kb; *ter*<sup>R/VP16 *trans*-activator, 1.2 kb with the *Mlu*I-*Xba*I probe. Figure 1 displays the locations of the restriction enzyme sites and probes. The stability of each viral gene in each packaging and producer cell line was examined by exposing the probed filters to phosphoimager plates for 18 hr. Densitometry was performed after scanning with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The band intensities of the early-passage cells were set at 1.0 and the band intensities of the late-passage cells were expressed as fractions thereof. Background was subtracted from all bands analyzed. All lanes were normalized for differences in loading by probing the filters with a 1.1-kb *Bgl*II-to-*Spe*I fragment of the human factor VIII cDNA to yield a band of 8.5 kb (GenBank accession number M14113).</sup></sup>

### Sodium butyrate treatment

Lentikat producer clones were plated in doxycycline (0.7  $\mu\text{g}/\text{ml}$ ) and at 90% confluence were induced with fresh growth medium minus doxycycline. Three days postinduction duplicate plates of each clone were refed with induction medium with or without 10  $\mu\text{M}$  sodium butyrate. Twenty-four hours later all plates were refed with fresh induction medium minus sodium butyrate. Medium was changed and collections taken on days 5–14. Conditioned medium collections were characterized for p24 and GFP titers.

### Assays for the detection of recombination

**Assay for replication-competent retrovirus.** To select the detector cells most permissive for amplification of R8.7 control virus, C8166, CEM-SS, SupT1 and HeLa T4 cell lines were tested. Cells of each cell type ( $2 \times 10^5$ ) were mixed with serially diluted stock of R8.7 virus to a final volume of 1 ml per well in 24-well plates. Infected cultures were incubated for 8–10 days, during which cells were split to avoid overconfluent culturing conditions. Cultures were scored for establishment of productive viral replication by monitoring syncytium formation and production of p24 antigen by enzyme-linked immunosorbent assay (ELISA). In spiking experiments the R8.7 virus stock was diluted into media containing variable amounts of vector generated by transient transfection. Median tissue culture infective dose (TCID<sub>50</sub>) determination was performed by the method of Reed and Muench.

**Assay for envelope-defective recombinant.** 293G detector cells were seeded in 6-well plates at  $5 \times 10^4$  cells per well in medium containing doxycycline 24 hr prior to addition of test samples. Cells were then washed with doxycycline-free medium, and test-conditioned media were added and incubated for 48 hr. Plates were refed with medium containing doxycycline and incubated for 2–3 weeks. Medium was replaced every 2–3 days and undiluted conditioned media were assayed for p24 in duplicate by immunocapture. To monitor the reproducibility of the assay detection limits serial dilutions of positive control R8.7ΔEnv viral stock were run in parallel with every assay.

### Treatment/infection of growth-arrested cells

HeLa cells were seeded at  $10^5$  cells per well of a 6-well plate and 2 hr after plating aphidicolin (15  $\mu\text{g}/\text{ml}$ ) was administered to cells. After 20–22 hr cells were infected with serial dilutions of either Lentikat2.54 population B-conditioned medium transferring the pHRCMVGFP expression cassette or transiently generated MuLV, VSV-G-pseudotyped vector transferring the 43.2GFP expression cassette (Finer *et al.*, 1994) in the presence of aphidicolin (15  $\mu\text{g}/\text{ml}$ ) and Polybrene (8  $\mu\text{g}/\text{ml}$ ). Twenty-four hours postinfection the cells were rinsed with Mg<sup>2+</sup>/Ca<sup>2+</sup>-free phosphate-buffered saline (PBS) and refed with 2 ml of HeLa growth medium. Cells were harvested after 72 hr and assayed for GFP expression by FACScan.

### Concentration of vector

Producer clones 14 and 262 were induced and 36 ml of each was harvested, filtered, and centrifuged in a Beckman (Fuller-

ton, CA) SW28 rotor at 19,500 rpm for 140 min (50,000  $\times g$ ). Supernatant was aspirated, tubes were inverted to drain off residual liquid, and pellets were resuspended in 1% fetal bovine serum (FBS) in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free PBS (FBS-PBS). To disrupt particle aggregates each suspension was shaken (lowest speed; table-top vortexer) at 4°C for 2 hr, and then diluted with FBS-PBS and centrifuged as described above. Supernatant was aspirated and the pellets were resuspended in 36  $\mu\text{l}$  of FBS-PBS, shaken for 2 hr at 4°C, and then flash frozen on dry ice and stored at -80°C.

### In vivo gene delivery

For vector injection, C57BL/6 mice were anesthetized with an intraperitoneal injection of tribromoethanol (1.25%; Sigma, St. Louis, MO) and positioned in a stereotactic frame (David Kopf Instruments, Tujunga, CA), and the skull was exposed by a small incision. One microliter of vector concentrate, previously resuspended by slow vortexing for 1 hr at room temperature and adjusted to a p24 concentration of 100 ng/ $\mu\text{l}$ , or vehicle only, was injected by a Hamilton (Reno, NV) syringe with a 33-gauge blunt-tip needle into the right hippocampus (stereotactic coordinates: AP -1.70, ML 2, and DV -2.5 from the skull surface) at a rate of 0.2  $\mu\text{l}/\text{min}$ . The needle was left in place for an additional 5 min before slow removal. At 4 and 16 weeks postinjection, mice were anesthetized and transcardially perfused with PBS followed by 4% paraformaldehyde–0.1 M sodium phosphate buffer, pH 7.4. The brain was removed, post-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 8–12 hr at 4°C, followed by a cryoprotective soaking in 30% sucrose at 4°C overnight. Brains were frozen in O.C.T. compound (Tissue-Tek, Sakura Finetek, Torrance, CA) on dry ice and 10- $\mu\text{m}$ -thick cryostat sections were cut and scored for GFP expression by direct fluorescence microscopy.

## RESULTS

### Generation of inducible packaging cell lines

Our strategy to obtain stable packaging cell lines for HIV-derived vectors was to limit expression of both the packaging and envelope constructs in producer cells to the time of vector collection by a tetracycline-regulated system. We first modified the minimal packaging construct pCMVΔR8.74 by replacing the CMV promoter with a minimal CMV promoter linked to tandem repeats of seven tetracycline operator sites (Tet<sup>o</sup>) as described by Gossen *et al.* (1995). We then deleted all HIV-1-derived sequence upstream of *gag*, except for the splice donor site, and optimized the sequence around the *gag* translation initiation site according to Kozak (1997) (Fig. 1). We derived the packaging clones from 293G cells (Ory *et al.*, 1996), which constitutively express the Tet<sup>R</sup>/VP16 trans-activator from the CMV promoter and also contain the VSV-G envelope gene under the control of a Tet-responsive promoter element, so that in medium containing tetracycline the VSV-G envelope is not expressed (Tet-off system) (Gossen *et al.*, 1995).

In two separate experiments 293G cells were cotransfected with a 9:1 DNA ratio of pTetCMVΔR 8.75 and pZeoSV2 and

TABLE 1. CHARACTERIZATION OF LENTIKAT PACKAGING CLONES FOR VIRUS PRODUCTION

Clone	Nontransduced		Transduced with GFP lenti vector					
	Non-induced p24 (ng/ml)	Induced p24 (ng/ml)	Non-induced p24 (ng/ml)	Total induced p24 <sup>a</sup> (ng)	Average induced p24 <sup>b</sup> (ng/ml)	Total titer <sup>c</sup> (TU × 10 <sup>6</sup> /ml)	Average titer <sup>b</sup> (TU × 10 <sup>6</sup> /ml)	Infectivity <sup>d</sup>
2.8	<LD <sup>e</sup>	24.60	0.01	360	9.0	2.50	0.06	6944
2.54	<LD	3.42	0.14	2958	42.25	27.39	0.39	9257
20.12	<LD	15.65	0.02	1236	30.90	7.75	0.19	6290
20.36	<LD	14.95	<LD	1755	35.10	3.08	0.06	1761
20.76	0.25	12.41	4.34	798	11.40	1.11	0.01	1375
20.81	<LD	19.80	0.01	1333	22.21	1.27	0.02	977
20.85	<LD	20.33	0.02	2109	52.70	20.48	0.51	9716
20.87	<LD	12.65	0.01	2550	36.42	25.23	0.36	9882
20.98	<LD	14.96	0.05	670	9.57	6.39	0.09	9552
20.104	0.2	184.71	0.19	2096	69.86	2.15	0.07	1025
20.116	<LD	29.71	0.34	765	25.48	2.08	0.07	2719
20.253	<LD	14.75	0.23	1706	56.85	8.45	0.28	4971

<sup>a</sup>Sum of p24 collected during induction of 10-cm plate, 10 ml of medium per plate.

<sup>b</sup>Sum of transducing units collected during induction of 10-cm plate, 10 ml of medium per plate.

<sup>c</sup>Average daily value over the 8-day collection.

<sup>d</sup>Total titer/total p24.

<sup>e</sup>LD, limit of detection. The limit of detection of p24 was 0.03 ng/ml.

clones were selected in the presence of zeocin (250 µg/ml) and tetracycline (1 µg/ml). We screened the clones for regulated production of vector particles and transduction efficiency of vector produced after introduction of a GFP transfer construct by infection. The culture medium of 366 clones was screened for expression of the mature Gag protein p24 by immunocapture assay before and after 3 days of induction in tetracycline-medium. Although 43 clones yielded p24 levels greater than 1 ng/ml postinduction, only 34 of these had nondetectable levels of p24 in the noninduced state. While clone 20.104 yielded p24 at 184 ng/ml on induction, most were in the 3 to 30-ng/ml range and 12 clones were chosen to test vector production.

The selected clones were infected twice with transiently produced vector containing a CMV-GFP expression cassette (Fig. 1) at an MOI of 5. Conditioned media from the transduced clones were collected prior to induction and at 24-hr intervals for 8 days after induction in tetracycline-free medium, and assayed for GFP transfer to HeLa cells by FACS and for particle output by p24 ELISA (Table 1). We found that the p24 induction curve was unique for each clone and that significant vector output was maintained over several days. Since there was no collection window that was common to all producer populations, the data in Table 1 are expressed as the total GFP transducing units (TU) and total nanograms of p24 collected per 10-cm plate after induction. The average titer, in TU per milliliter of culture medium, and vector infectivity are also shown. Vector infectivity, defined as the ratio of TU to physical particles measured as nanograms of p24, gives a reliable measure of the potency of a vector preparation. The data in Table 1 show that levels of p24 from the initial screen were not predictive of vector production. This could also be expected from the variability of the production kinetics after withdrawal of tetracycline. Five of the 12 producer populations made better than 10<sup>5</sup>

TU/ml, and three of those populations (2.54, 20.85, and 20.87) had cumulative titers of 2 × 10<sup>7</sup> GFP transducing units and infectivity levels approaching those seen in transient transfections. The good infectivity of vector produced by these clones indicated the viability of our strategy to build stable packaging cells.

In the course of these experiments we noted that the conditioned medium from some producer populations, while apparently allowing for a high level of GFP transfer, could not be titrated by serial dilution in the HeLa assay, and that GFP expression in cells transduced by these media decreased over time. This pseudotransduction was possibly dependent on the excess GFP protein expressed in producer cells. In contrast, GFP expression in target cells for the other clones was dependent on the vector dose and remained constant with time. Accordingly, all target cells were maintained in culture for at least 10 days prior to analysis to verify transduction.

#### Generation of vector producer clones

The packaging cell line Lentikat 2.54 was selected for further characterization to determine its maximum potential for vector output and its stability in long-term culture. As the expression level of the transfer vector RNA is a crucial limiting factor for the titer of vector produced by transient transfection (Dull *et al.*, 1998), we added vector genomes to clone Lentikat 2.54 by serial rounds of infection with transiently produced GFP vector (VSV-G pseudotyped) for a total of five infections (populations A to E). After each infection a portion of the population was set aside for characterization before the next infection was performed. FACS analysis proved that each round of infection increased the average level of transgene expression and consequently, vector copy number, in each producer cell

(Fig. 2A). Populations from infections B to E were then simultaneously induced and the conditioned media collected for 8 days and assayed as described above (Fig. 2B and C). We found that each new round of infection produced an increase in the titer of output vector. Infection E titer peaked at  $6.6 \times 10^6$ /ml, and remained over  $10^6$ /ml for at least 6 days. Infectivity of vector also increased with each infection and reached levels comparable and superior to those obtained by transient transfection. We also noted that the kinetics of induction of particle release (measured by p24) were faster in the transduced clones, reaching higher maximal levels and yielding a higher total output in the collection window when compared with the values observed for the original nontransduced Lentikat2.54 cells (Fig. 2C). We ruled out the outbreak of replication-competent and replication-defective recombinants, as shown below. Similar increases in p24 output were observed in other packaging clones after transduction of the transfer vector (see Table 1; and data not shown). The possible reasons for the increased p24 output after vector transduction are discussed below.

We then isolated individual producer clones from population E by limiting dilution for further characterization. We were surprised to find that of the 286 clones assayed, only 66 had induced p24 levels above 1 ng/ml, which corresponded to the 66 clones with HeLa titers of greater than  $10^5$ /ml. Only 23% of the clones had retained p24 expression, which suggested that the population was highly unstable or not clonal. We then subcloned an early passage of Lentikat2.54 and probed 24 clones for HIV-1 *gag-pol* sequences by polymerase chain reaction (PCR) and found that only 7 of the 24 clones were positive, suggesting that the instability or oligoclonality was a feature of the original Lentikat2.54 packaging cell line isolate. We derived and characterized new producer cell populations from individual *gag-pol*-positive subclones of Lentikat2.54 cells (not shown). These experiments reproduced the above-described results in satisfactory yield of infectious vector, and increased titer with increasing round of vector infection into the producer cell population. Ongoing studies will clarify whether the new subclones have gained an advantage in stability.

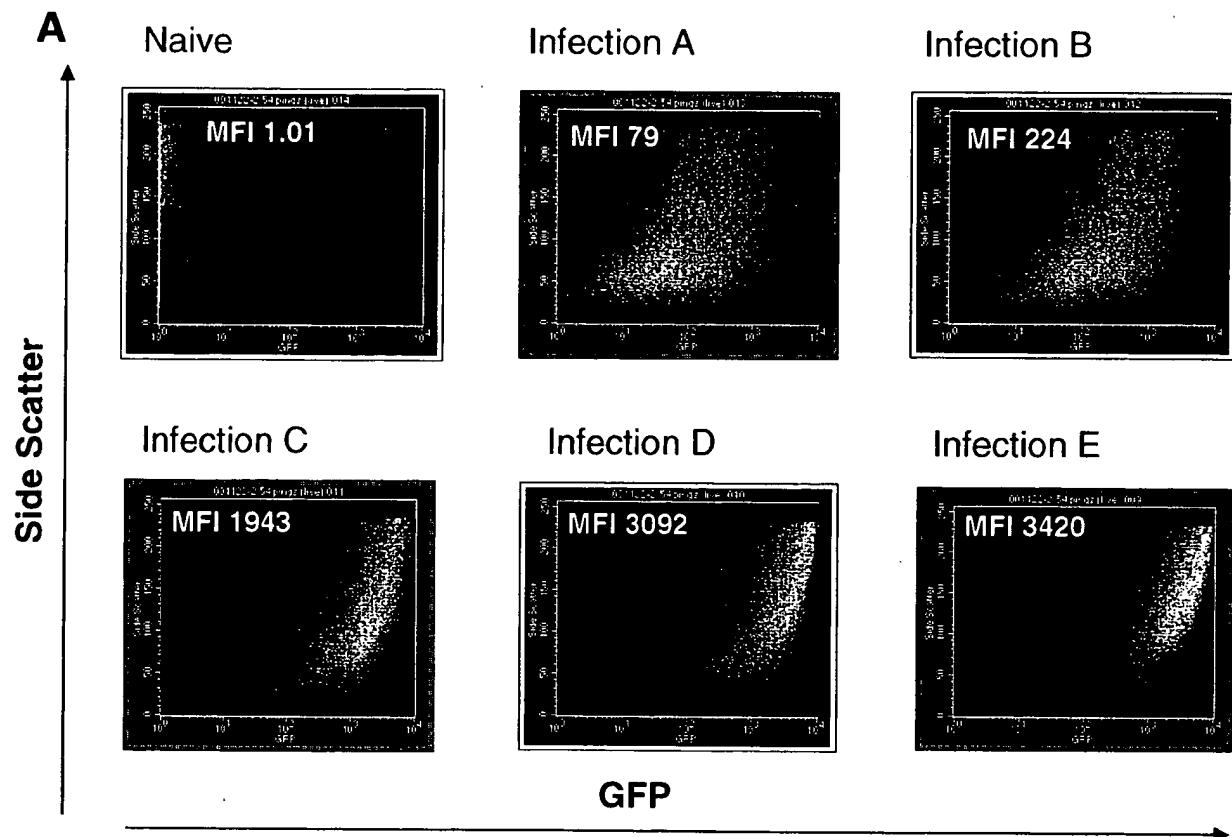
#### Stability in culture of vector producer clones

It was then important to assess the stability of the individual producer clones. The parental Lentikat2.54, the vector producer population E, and 10 vector producer clones with induced titers in excess of  $10^6$ /ml were grown in culture for 12 weeks in the presence of doxycycline. At 4-week intervals cells were removed from the cultures and induced in doxycycline-free medium. The use of doxycycline rather than tetracycline delayed the induction windows by several days. Media were collected at 24-hr intervals for at least 9 days and assayed for titer and p24. Total titer and p24 production were calculated for days 5–9 (Fig. 3). As expected from the subcloning experiments described above, producer population E was highly unstable: after 12 weeks in culture the induced particle output and titer had dropped significantly from a total production of 6200 ng of p24 to only 400 ng of p24, and total transducing units had fallen 50-fold from  $5.7 \times 10^7$  to  $1 \times 10^6$  TU. We found, however, that despite the instability of the parental population, 5 of the 10 producer clones were stable (clones 14, 68, 223, 262, and 281) over the 12 weeks of the experiment. Clones 16, 104, 244,

and 260 had lost titer and p24 output by passages 8 and 12, and clone 111 appeared to be in decline. We observed that clones that maintained particle production also maintained titer, and that initial p24 values were in the same range for all stable clones.

To identify possible reasons for the instability observed in half the producer clones we monitored by Southern analysis the maintenance of the different constructs introduced into the packaging and producer cells. Genomic DNA was prepared from each of the parental cell lines 293 and 293G, from Lentikat2.54, the vector producer population E, two stable producer clones (14 and 262), and one unstable clone, 244 (Fig. 4). DNA was sampled from noninduced cells after short (week 1) or long-term culture (week 14), and from cells induced for 9 days after long-term culture. All samples were digested with *Sst*I, electrophoresed on an agarose gel, Southern blotted onto nylon filters, and scored by radiolabeled probes specific for the sequences in pTetCMV $\Delta$ R8.75, VSV-G, GFP, Tet<sup>R</sup>/VP16 *trans*-activator, and the endogenous factor VIII gene. Restriction by *Sst*I was selected because it released well-recognized fragments from the original constructs, and because theoretical recombinants between transfer and packaging vectors would maintain two *Sst*I sites and release a fragment of a size similar to that labeled by the *pol* probe from pTetCMV $\Delta$ R8.75 (Fig. 1). In this way we could screen for either the loss or the possible amplification of *gag-pol* genes by a recombinant spreading in the culture. The expected bands were produced for all four types of DNA: VSV-G at 6.6 kb, TetCMV $\Delta$ R8.75 at 4.7 kb, GFP at 1.4 kb, and Tet<sup>R</sup>/VP16 *trans*-activator at 1.2 kb. In addition, by probing downstream of the 3' *Sst*I site in TetCMV $\Delta$ R8.75 with the *Bam*HI-*Xba*I probe we were able to demonstrate a single, identical integration event of the packaging vector (data not shown). This 6.1-kb band was common to Lentikat2.54 and all its descendants. However, the intensity of this common band was less in Lentikat2.54 than it was in population E and the producer clones. All bands were evaluated by PhosphorImager analysis after normalizing for DNA loading with an 8.5-kb band generated by a probe specific for the endogenous factor VIII gene. For each cell line, the density of each band was compared with the value at passage 0 in order to detect differences in copy number after 14 weeks of continuous culture and/or after 9 days of induction.

In all cases, except that of Lentikat2.54, the bands corresponding to the *gag-pol* genes appeared to be stable, and thus there was no detectable evidence of either amplification or loss of *gag-pol* during the course of 14 weeks of culture and induction. We consistently observed that the *gag-pol* copy number for Lentikat2.54 was less than that of the producer clones and that it varied with long-term culture, which supported our earlier observation that the original isolate of this cell line was not clonal. The GFP transfer vector also appeared to be stably integrated. However, the Southern analysis showed that the VSV-G gene was lost during culture, and this loss correlated with those clones or populations that lost titer during passage. Lentikat2.54, population E, and clone 244 all lost VSV-G DNA. Clones 14 and 262 appeared stable in doxycycline, but lost a small fraction of VSV-G DNA during the induction. Instability of VSV-G DNA was also reflected in its different content among the cell populations tested at the initial time of the experiment. An interesting observation is that those cells that lost



**FIG. 2.** Analysis of Lentikat2.54 packaging clone before and after serial infection with a GFP transfer vector. Lentikat2.54 was infected serially five times (A to E) with VSV-G-pseudotyped HR2CMVGFP transfer vector. Nontransduced Lentikat2.54 and transduced populations A–E were analyzed by flow cytometry for GFP expression (MFI, mean fluorescence intensity of GFP) (A), populations B–E were induced in tetracycline-free growth media, and the conditioned media analyzed daily (B) for endpoint titer on HeLa cells (TU/ml; columns) and infectivity (TU/ng p24; lines). (C) Total yield of particles, in nanograms of p24, and of infectious vector, in transducing units (TU), and average particle concentration (ng p24/ml), titer (TU/ml), and particle infectivity (TU/ng p24) for the induction period.

VSV-G DNA were also the same that lost p24 expression, although the pTetCMVΔR8.75 DNA appeared to be intact by Southern analysis. This could be because the expression plasmid for VSV-G was cotransfected with the plasmid expressing the Tet<sup>R</sup>/VP16 *trans*-activator during generation of 293G cells (Ory *et al.*, 1996). Since the DNA incorporated in stable transfectants is often concatemeric, we postulated that a selective pressure against the VSV-G DNA would also coselect against the *trans*-activator gene and lead to loss of both genes and, consequently, lack of expression of the pTetCMVΔR8.75 DNA. Therefore we ran the same samples on a second Southern blot, probed for the Tet<sup>R</sup>/VP16 *trans*-activator, and found a pattern identical to that of VSV-G, thus demonstrating the loss of both VSV-G and *trans*-activator genes in the unstable clones and populations (Fig. 5), leading to the lack of expression of the pTetCMVΔR8.75 DNA.

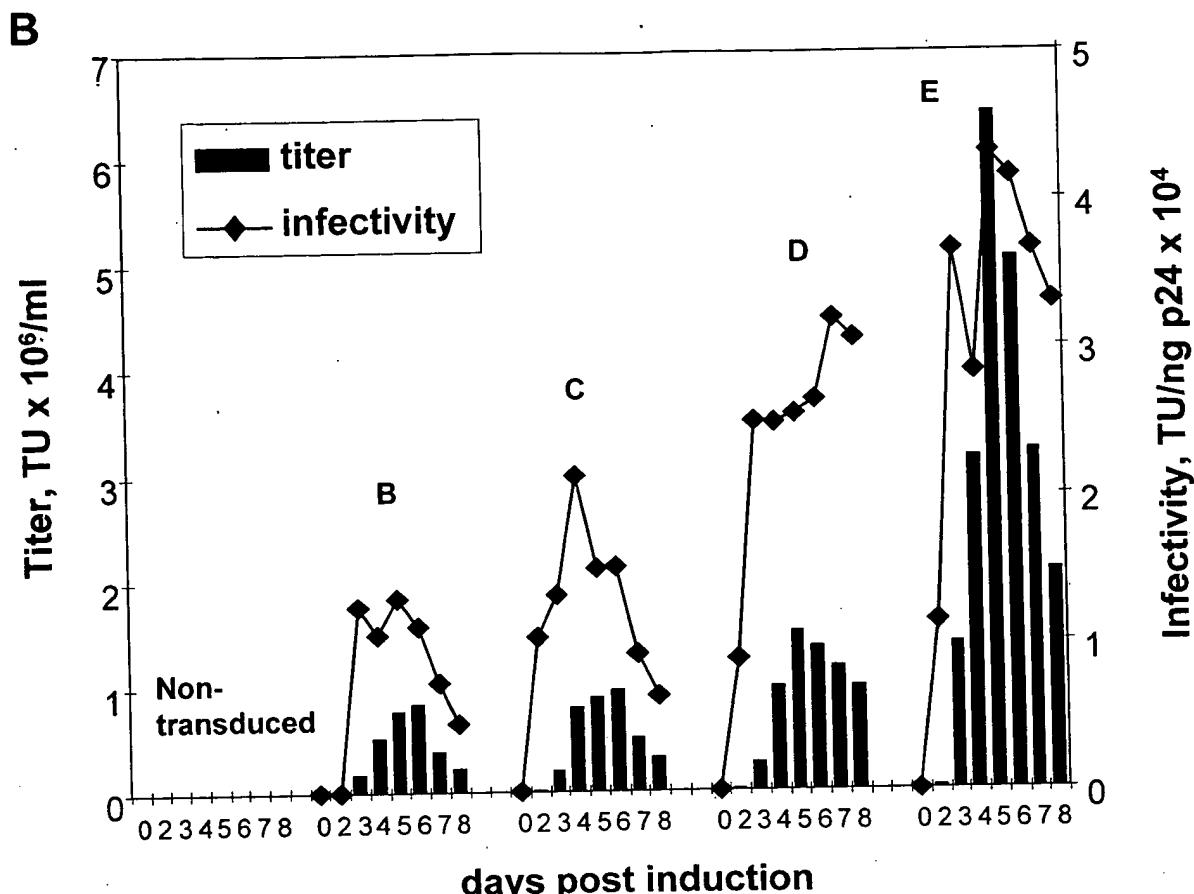
We also tested whether silencing of transcription from the transfected DNA was responsible for some of the decay in performance of the producer clones. It was previously reported that treatment with sodium butyrate reactivated transcription from Tet-responsive promoters after long-term culture of 293 cells expressing a first-generation packaging construct for lentiviral

vectors (Kafri *et al.*, 1999). Butyrate treatment did not rescue vector output from our clones that had lost it. In the clones that had maintained output the treatment changed the kinetics of induction by shortening the induction time, but did not improve the total yield of vector (Fig. 6).

These results indicate that clonal variation allows the isolation of stable vector producer clones with satisfactory output from Lentikat2.54 cells. Moreover, the instability of vector producer clones appeared to be an intrinsic feature of the parental cell line from which they were derived, and may not be necessarily linked to the design or type of vector. Our data also point to possibly more effective strategies for building stable packaging cell lines that aim at improving the stringency of VSV-G expression control, removal of counterselective pressure, and sequential introduction of the different types of DNA.

#### Screening for recombination of vector and packaging constructs

To screen our vector producer cells for the absence of replication-competent recombinants (RCRs) we needed to establish an assay in which we could incubate aliquots of the conditioned

**C**

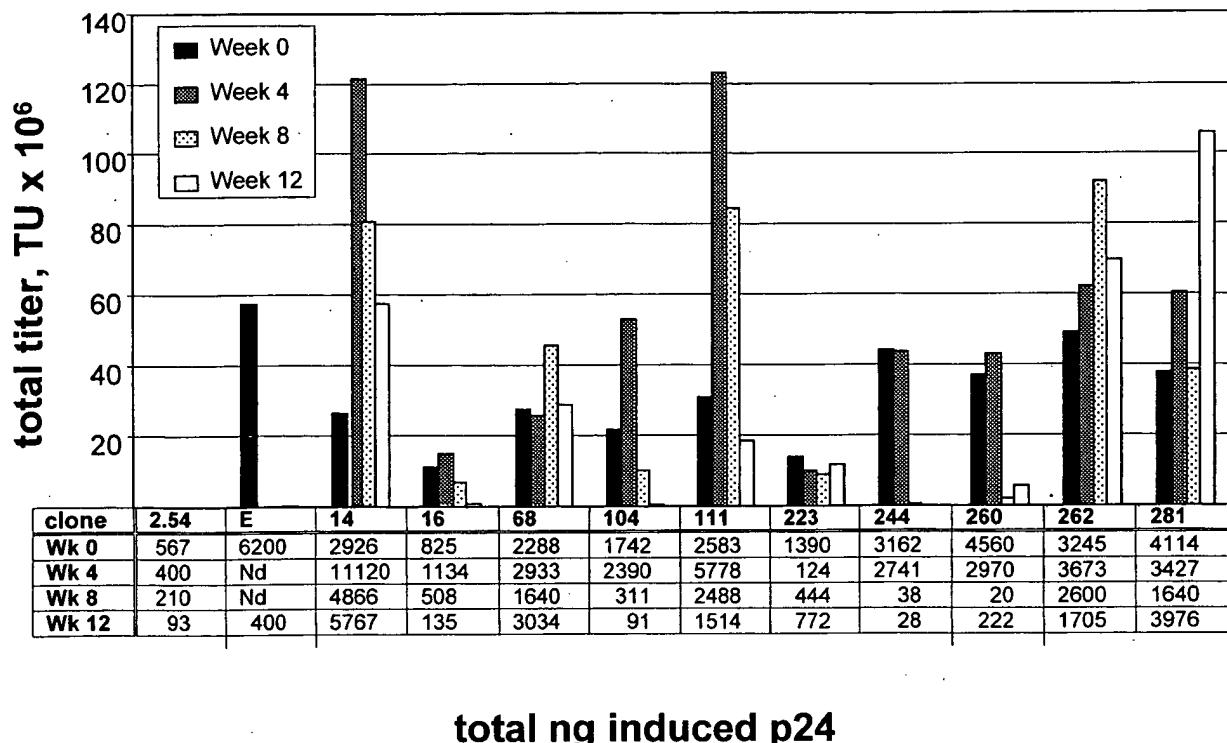
Infection	Non-induced		Induced					
	P24 ng/ml	Titer <10 <sup>4</sup> TU/ml	Total ng p24 (1)	Average induced p24 ng/ml	Total titer, TU x 10 <sup>6</sup> (2)	Average titer, TU x 10 <sup>6</sup> /ml	Infectivity (3)	
Non-transduced	0.01	0	729	12.15				
B	0.12	0	2958	49.20	27.39	0.46	9257	
C	0.57	0	2777	46.29	36.67	0.61	13201	
D	0.00	0	2295	38.24	62.65	1.04	27261	
E	0.27	0	5694	94.91	212.88	3.55	37351	

(1) Sum of p24 collected during induction of 10cm plate, 10mls media per plate.

(2) Sum of transducing units collected during induction of 10cm plate, 10mls media per plate.

(3) Total titer / total p24

FIG. 2. *Continued.*



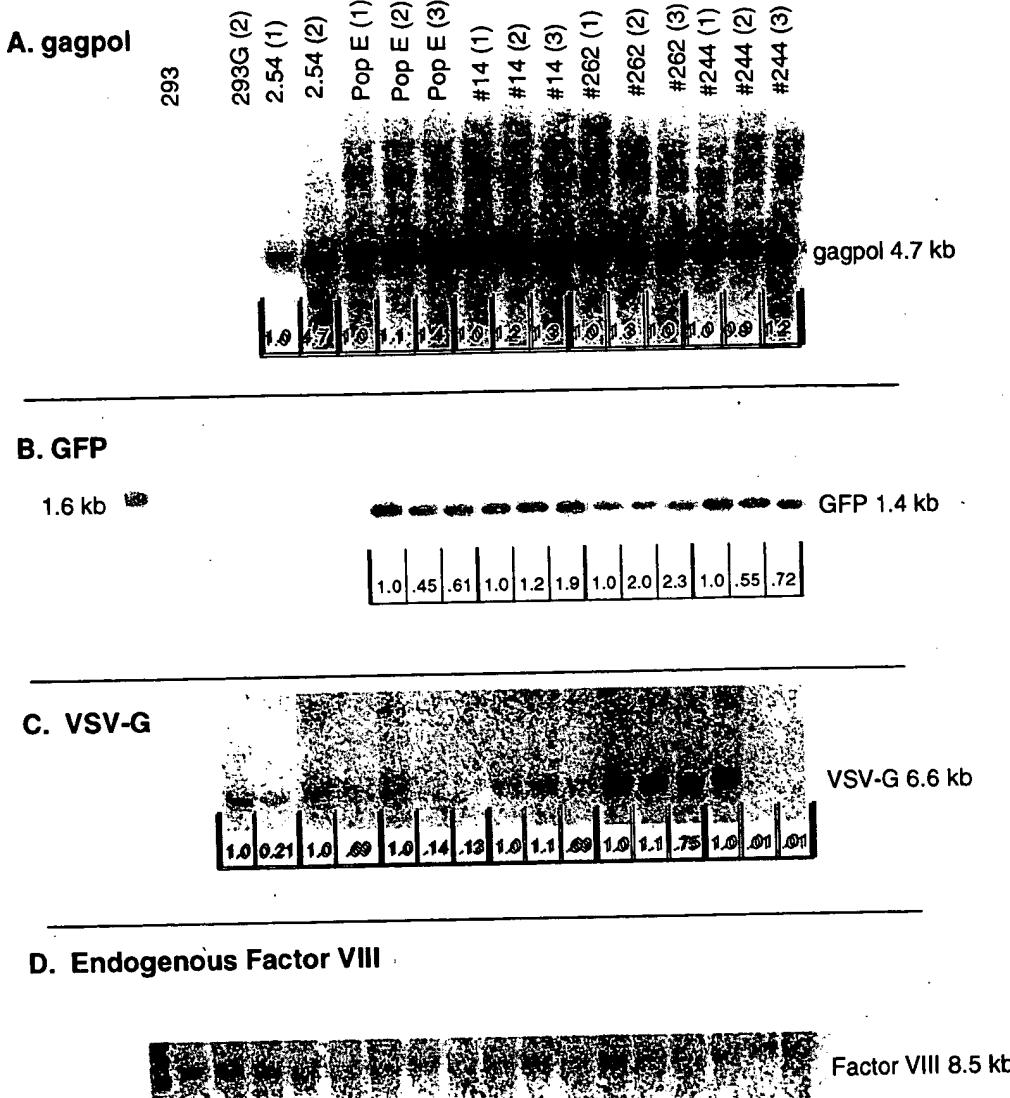
**FIG. 3.** Characterization of Lentikat2.54 producer clones for stability of vector output with time. Ten producer clones of GFP vector (indicated by the clone number) and 2 controls (Lentikat2.54 and transduced parental population E) were induced at 4-week intervals by removal of doxycycline. Medium was collected at 24-hr intervals and analyzed for end-point transduction of GFP into HeLa cells (bar graph) and for the content of p24 (chart). Data are expressed as the total yield of p24 and transducing units for the induction period of each clone. Nd, Not done.

medium with a cell line shown to be highly permissive to HIV-1 infection and then monitor p24 released in the culture supernatant. Cultures would be grown for a minimum of 3 weeks for clearance of the input p24 and amplification of any RCR. For a positive control RCR that mimicked one derived from the minimal packaging construct introduced into Lentikat2.54 cells, we deleted the four accessory genes of an infectious clone of HIV-1, R8 (Gallay *et al.*, 1995), and cotransfected this plasmid with the expression construct for VSV-G into 293T cells to match the conditions of vector production for the initial infection. After the first round of infection this attenuated *vpr*<sup>-</sup> *vpu*<sup>-</sup> *vif*<sup>-</sup> *nef*<sup>-</sup> HIV-1 clone, referred to as R8.7, replicated by its own envelope. This approach was dictated by the biosafety concerns of generating a genotypic VSV-G-pseudotyped HIV-1. On the other hand, the adoption of a multiply attenuated packaging construct for vector production reduced correspondingly the biohazard of any possible RCR, including that represented by growing the positive RCR control even when phenotypically pseudotyped by VSV-G. The R8.7 virus grew efficiently in several tested cell lines, and as expected from its multiply deleted genome (Cullen, 1998), it did not grow detectably in primary human lymphocytes (data not shown).

Among the target cell lines tested, the human T cell line C8166 supported virus replication most efficiently as evidenced

by the fact that the minimal infectious dose required to establish productive infection of the culture was at least one order of magnitude lower than for any other cell line tested (data not shown). Consistent with our previous observation that VSV-G-pseudotyped vector particles were found to be significantly more infectious than their counterparts incorporating the gp120 glycoprotein of HIV-1 (Bukovsky *et al.*, 1999), we found that the minimal viral infectious dose was lowered another 1000-fold when detector cell cultures were infected with R8.7 particles pseudotyped with VSV-G (data not shown). The TCID<sub>50</sub> of C8166 cells by VSV-G-pseudotyped R8.7 virus was 1 fg of p24 equivalent of virus, as determined by the method of Reed and Muench (data not shown). Assuming that each virion contains approximately 2000 molecules of p24 capsid protein and that these molecules are assembled in particles of relatively homogeneous size, this would correspond to approximately 12 physical particles, indicating the high sensitivity of this assay. In spiking experiments we also established that the addition of a nonreplicating control vector at a maximum MOI of 5–10 did not increase the median infective dose (ID<sub>50</sub>) of the positive control (data not shown). Thus we used these experimental conditions to screen vector batches for RCR. To date we have not found a positive sample (more than 60 samples from more than 30 producer cell cultures were tested one or two times).

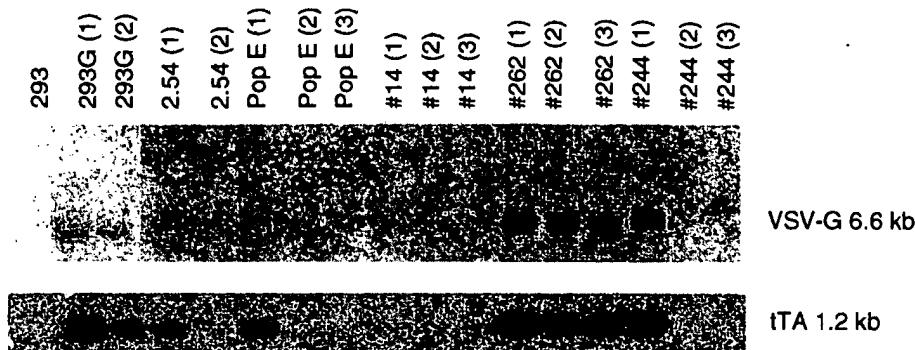
## LENTIVIRAL VECTOR PACKAGING CELL LINE



**FIG. 4.** Analysis of the genetic stability of vector packaging, transfer, and envelope sequences in the packaging and producer cell lines. Southern analysis of DNA from the indicated cells grown in culture for 1 week (1), 14 weeks (2), and 14 weeks plus 1 week of induction (3), digested with *Sst*I and probed for (A) *gag-pol* (B) GFP, (C) VSV-G, and (D) endogenous factor VIII gene sequences, respectively. The densitometry of the resulting bands after PhosphorImager analysis is displayed at the bottom of (A-C), expressed as fractions of the early-passage band intensities for each cell line. Band sizes and the 1.6-kb molecular weight marker are indicated.

We also developed a sensitive assay for the detection of recombinants between packaging and transfer vector constructs that are able to transfer the viral *gag-pol* genes but lack an envelope gene (A. Bukovsky and L. Naldini, in preparation). In this assay aliquots of the vector batch were incubated with 293G cells, which were concurrently induced to express the VSV-G envelope. By supplying the envelope *in trans* these cells allowed amplification of a recombinant expressing only the HIV-1 *gag-pol* genes. The culture medium was changed every 2–3 days and assayed for p24. If the vector preparation were contaminated by recombinants productive for *gag-pol* transfer,

subsequent collections would have increasing amounts of p24. A positive control sample for the theoretical recombinant was generated by cotransfected an envelope-defective derivative of the pR8.7 plasmid described above, called pR8.7ΔEnv, with the VSV-G expression plasmid. The sensitivity of the assay was established by adding serial dilutions of the engineered recombinant. As shown in Fig. 7A, the assay followed the expected kinetics: the lower the input of recombinant added to the assay, the longer it took to amplify. The assay amplified a recombinant input as low as 6 fg of p24 to detectable levels starting at day 10, while an input of 0.6 fg of p24 remained negative even



**FIG. 5.** Concomitant loss of VSV-G and  $\text{Tet}^R/\text{VP16}$  *trans*-activator DNAs with long-term culture of vector packaging and producer cells, leading to the lack of expression of the packaging construct. Southern analysis of  $\text{SstI}$ -digested DNA from the indicated cells grown in culture for 1 week (1), 14 weeks (2), and 14 weeks plus 1 week of induction (3). The upper panel was probed for VSV-G sequences, and the lower panel for the  $\text{Tet}^R/\text{VP16}$  *trans*-activator. Band sizes are indicated.

after 16 days. The limit of detection of p24 by the immunocapture assay was 2.5 pg/ml. To reproduce more accurately the actual conditions encountered in an assay, dilutions of the R8.7 $\Delta$ Env recombinant were added in the presence or absence of excess vector generated by transient transfection (Fig. 7B). This did not alter the sensitivity of the assay. The conditioned media from LentiKat2.54 and all 10 producer clones induced at passage 12, were then examined for contamination by recombinants expressing the HIV-1 *gag-pol* genes. In all cases we observed a steady decline in p24 levels in the culture. Figure 7C shows a representative panel of clones and the decline in p24 levels from day 5 to day 16 of the assay. Considering the amount of vector tested and the limit of detection of the assay, we can conclude that the vector batches tested contained less than 1 particle in the  $1.6 \times 10^7$  tested containing a recombinant between transfer and packaging construct capable of transferring *gag-pol* packaging functions to target cells.

#### Performance of vector from stable producer cells

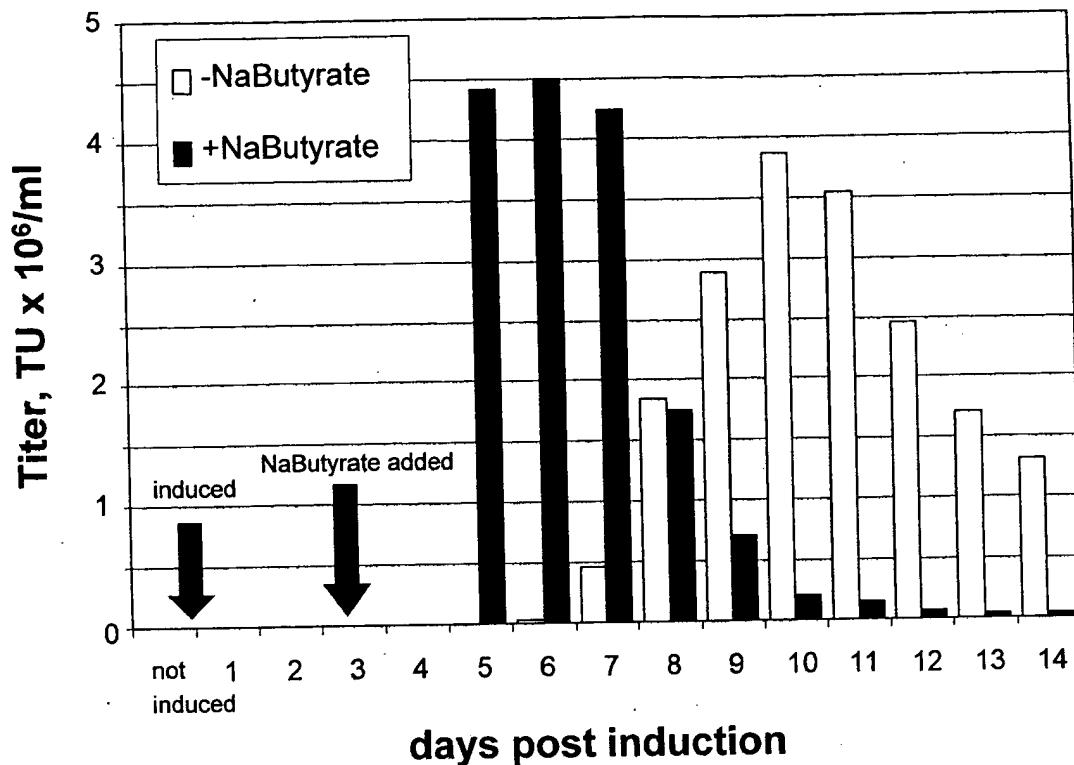
Having established the safety of the stable packaging system we wanted to functionally characterize the output vector to verify that it performed equivalently to that produced by transient transfection. Conditioned media from LentiKat2.54 producer population B were evaluated for their ability to transduce growth-arrested cells (Naldini *et al.*, 1996a). HeLa cells were treated with aphidicolin (15  $\mu\text{g}/\text{ml}$ ) 24 hr prior to infection with population B conditioned medium or a VSV-G-pseudotyped MuLV vector delivering GFP. Growing HeLa cells were readily transduced with vectors made either by a lentiviral or oncoretroviral core, whereas the aphidicolin-treated cells were transduced only by the lentiviral vector in the population B conditioned medium. In addition, we demonstrated that the conditioned media from producer clones 14 and 262 could be efficiently concentrated to obtain vector stocks displaying high titer and transduction frequency. Clone 14 and 262 conditioned media were concentrated 1000-fold to titers reaching  $1 \times 10^9$  TU/ml, with an average recovery of 80%. When p24-matched

doses of vector before and after concentration were used to transduce HeLa cells we observed similarly high transduction frequencies by FACS analysis, indicating that the concentration step increased the transduction efficiency without concentrating contaminants that interfere with transduction (data not shown).

The concentrated vector stock from clone 14 was tested for efficient delivery *in vivo* of the GFP expression cassette. One microliter of the stock was injected into the brain of C57BL/6 mice, into the right hippocampus, under stereotactical guidance. Four and 16 weeks after the injection, mice were killed and 10- $\mu\text{m}$ -thick cryostat sections were cut from the brain and analyzed for GFP expression by direct fluorescence microscopy. As shown in Fig. 8, remarkable levels of transduction of cells with the morphological features of hippocampal neurons were demonstrated in the injected side of the brain, as previously shown for vector produced by transient transfection (Naldini *et al.*, 1996b).

#### DISCUSSION

We have successfully generated and characterized a stable packaging cell line for HIV-1-based vectors. The minimal HIV-derived packaging construct introduced into the cells allowed for safe production of vector. By serial infection of the transfer construct, producer clones could be obtained yielding vectors with titer, infectivity, and performance matching or exceeding those of vector obtained by transient transfection. The titration of vector genomes by serial transduction of the packaging cell line indicated that, similar to what was observed in transient transfection, the level of expression of vector RNA in producer cells is a major rate-limiting factor for the transduction efficiency of the output vector. Furthermore, the achievement of optimal vector infectivity indicates that the packaging cell line did not produce excess defective particles. This was shown by the nearly quantitative concentration of the vector to high titers allowing efficient gene transfer *in vivo*, such as into



**FIG. 6.** Sodium butyrate treatment of Lentikat vector producer clone 14. Cells were induced on day 1 by refeeding with doxycycline-free medium, and treated with (solid columns) or without (open columns) 10  $\mu$ M sodium butyrate for 24 hr on day 3. Conditioned media were analyzed daily for end-point transduction of GFP into HeLa cells.

hippocampal neurons after injection of the vector into the mouse brain.

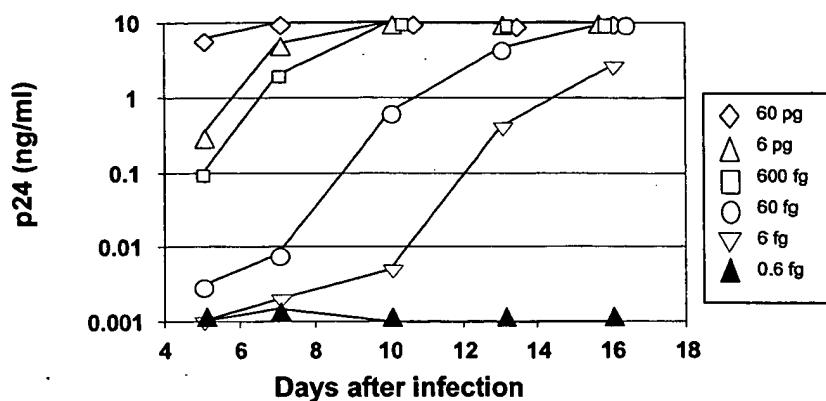
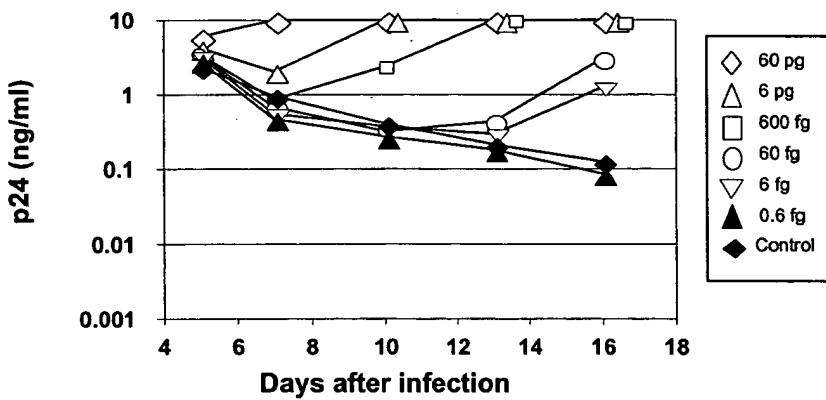
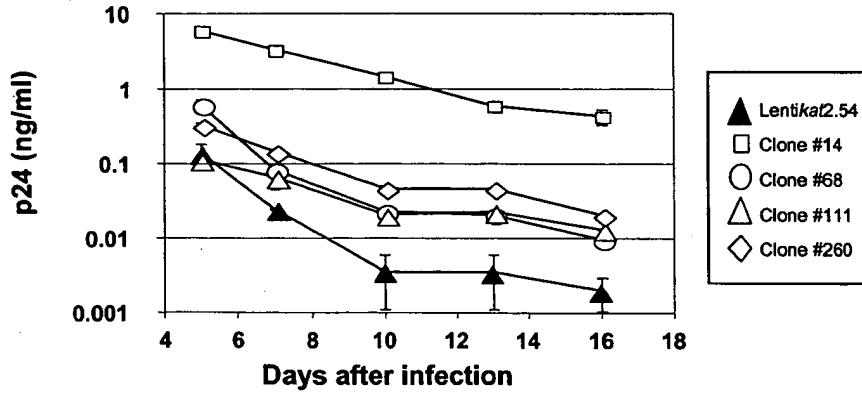
The packaging cell line was derived from 293G cells (Ory *et al.*, 1996). Some of the advantages of the 293 host cell are the human origin, good transfectability and adaptation to growth in suspension, a safe track record in the production of retroviral vectors (Burns *et al.*, 1993; Pear *et al.*, 1993; Finer *et al.*, 1994; Davis *et al.*, 1997; Yang *et al.*, 1999), and moderate dependence on the accessory genes *vpu*, *vif*, and *nef* of HIV-1 to generate infectious virus.

An unexpected observation was that the kinetics of induction and the total output of vector particles increased after transduction of the vector construct into the packaging cells. There are several possible explanations for this phenomenon. We ruled out that the observed increases were due to an amplification of the packaging DNA in the producer population, as it could occur by the outbreak and spread of a recombinant between the transfer and packaging construct. It is possible that the RNA-binding activity of Gag proteins responsible for encapsidation of the viral genome interferes with efficient translation, both *in cis* and *in trans*. A residual interaction could occur between the Gag protein and its mRNA in packaging cells, particularly in the absence of RNA carrying an intact encapsidation signal. When the transfer vector RNA is titered up into packaging cells by serial transduction it may progressively out-

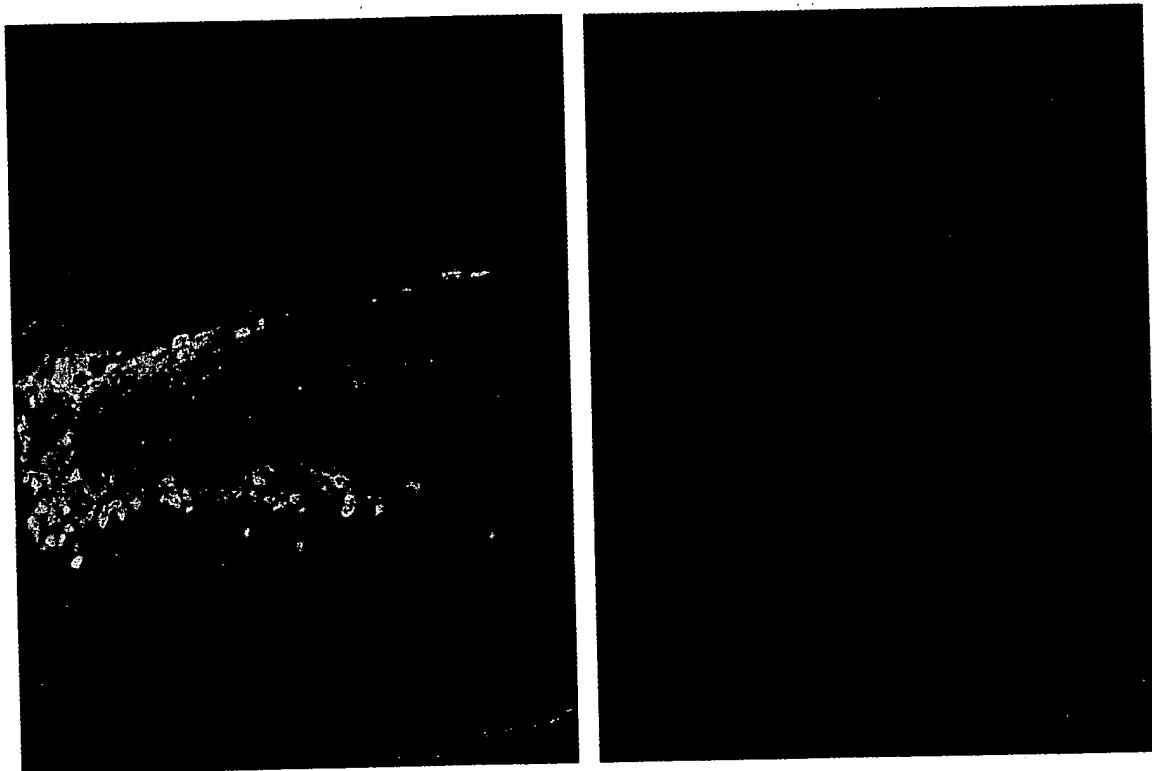
compete the Gag mRNA from binding sites in the Gag proteins, and increase its translational output (A. Bukovsky and L. Naldini, unpublished observation).

An important safety issue was raised by the use of the VSV-G envelope in stable producers. We showed that cells expressing high levels of VSV-G do not exhibit interference with superinfection, that is, they could be infected efficiently by VSV-G-pseudotyped vector or virus. Self-infection of a producer clone is one undesirable feature that may compromise its stability and increase the risk of generation of recombinants even by such unlikely mechanisms as gene capture. The vector producer system that we have developed has a built-in mechanism that limits self-infection. Expression of the packaging and envelope functions to adequate levels for release of infectious particles is limited to a short window of time after induction. While we could not detect infectious vector in the noninduced state, we observed, however, a basal level of expression of the VSV-G protein. This "leaky" expression appeared significant enough to induce a selective pressure against the maintenance of the VSV-G DNA in long-term culture, and it was a major factor in limiting the stability of the cell line. It thus appears that a more stringent control of VSV-G expression would improve the reliability and performance of stable packaging cell lines.

The packaging cell line and all vector producer clones de-

**A. R8.7ΔEnv****B. R8.7ΔEnv + Control Vector****C. Vector producing clones**

**FIG. 7.** Assay for replication-defective recombinants that transfer HIV-1 *gag-pol* packaging functions to target cells. 293G reporter cells were induced and exposed to control and test vector samples from the indicated Lentikar2.54 producer clones. Conditioned media from the assay were monitored for p24 levels at designated intervals. Assay sensitivity: (A) reporter cells were infected with the indicated serial dilutions of the positive control vector R8.7ΔEnv, VSV-G pseudotyped, as described in Materials and Methods. The envelope-defective recombinant was amplified efficiently, starting from the inoculum of 6 fg of p24. (B) The same dilutions of VSV-G-pseudotyped R8.7ΔEnv shown in (A) were spiked into lentiviral GFP vector (200 ng of p24) produced by transient transfection. The assay sensitivity was unchanged in the presence of excess vector particles. Note that the slow clearance of input p24 antigen does not prevent detection of amplifying spiked recombinant. (C) Test of vector samples. Reporter cells were exposed to 1 ml of the conditioned medium from the indicated late-passage, induced Lentikar2.54 and producer clones. No p24 amplification was observed. The limit of detection of p24 by the immunocapture assay was 2.5 ng/ml.



**FIG. 8.** Concentrated vector stock from Lentikat cells efficiently transduces cells with the morphological features of hippocampal neurons after direct delivery into the mouse brain. Cryostatic sections from the hippocampus of a GFP vector-injected (*left*) and control-injected (*right*) mouse were examined 4 months after injection for GFP fluorescence. Similar results were obtained in four mice examined 4 and 16 weeks postinjection.

scribed here were shown to be free from replication-competent recombinants by a highly sensitive assay. However, the outbreak of a replication-competent recombinant is a remote event given the hybrid composition and the split design of the lentiviral vector system. While this is a crucial safety feature of this type of vector, it also makes it difficult to compare the quality of different vector batches and different vector production systems. We thus developed an assay to monitor the appearance of recombinants between packaging and vector constructs that transfer the viral *gag-pol* genes without having to rely on the extremely unlikely outcome of an RCR outbreak. Such recombinants are replication defective but they may be propagated in vector producer cells that do not exhibit envelope-dependent interference with superinfection as is the case with VSV-G. The recombinants would interfere with transduction of the vector construct because they would compete for encapsidation by the viral particles. Moreover, they may be harmful to a vector recipient as they could transfer vector-packaging functions, and may cause toxicity or immune reactions. Most importantly, the possible amplification of such a recombinant in a producer cell line increases the risk of additional recombination events eventually leading to generation of a replication-competent retrovirus. Sensitive detection of envelope-

defective recombinants expressing the viral *gag-pol* genes is thus crucial to validate and maintain the performance of a vector producer system, and to prove the purity and safety of a vector batch. The packaging cell line described here and all vector producer clones derived from it did not yield any detectable recombinants of such a type.

An additional improvement in the safety of vector production could be achieved by a self-inactivating (SIN) transfer vector (Miyoshi *et al.*, 1998; Zufferey *et al.*, 1998; Iwakuma *et al.*, 1999). This would not only reduce the risks associated with re-infection of the producer system, but also enhance the safety of the output vector, alleviating the risks of recombination or mobilization in the recipient (Bukovsky *et al.*, 1999). However, as SIN vectors cannot be infected to generate producer cells, obtaining a good producer clone may require the screening of a large population of transfectants, or the adoption of other strategies such as targeted recombination of a preselected vector integration site (Vanin *et al.*, 1997), or the use of self-maintaining episomal constructs (Kinsella and Nolan, 1996).

The second-generation packaging clone described in this work, and the assays developed for its characterization, should enable scaling-up and advancing the applications of gene transfer mediated by lentiviral vectors.

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Address reprint requests to:

Dr. Luigi Naldini

Laboratory for Gene Transfer and Therapy  
IRCC, Institute for Cancer Research and Treatment  
University of Torino Medical School  
Strada Provinciale 142  
10060 Candiolo (Torino), Italy

E-mail: lnaldini@ircc.unito.it

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# Alternative Splicing of Human Immunodeficiency Virus Type 1 mRNA Modulates Viral Protein Expression, Replication, and Infectivity

DAMIAN F. J. PURCELL\* AND MALCOLM A. MARTIN

Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892

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Multiple RNA splicing sites exist within human immunodeficiency virus type 1 (HIV-1) genomic RNA, and these sites enable the synthesis of many mRNAs for each of several viral proteins. We evaluated the biological significance of the alternatively spliced mRNA species during productive HIV-1 infections of peripheral blood lymphocytes and human T-cell lines to determine the potential role of alternative RNA splicing in the regulation of HIV-1 replication and infection. First, we used a semiquantitative polymerase chain reaction of cDNAs that were radiolabeled for gel analysis to determine the relative abundance of the diverse array of alternatively spliced HIV-1 mRNAs. The predominant *rev*, *tat*, *vpr*, and *env* RNAs contained a minimum of noncoding sequence, but the predominant *nef* mRNAs were incompletely spliced and invariably included noncoding exons. Second, the effect of altered RNA processing was measured following mutagenesis of the major 5' splice donor and several cryptic, constitutive, and competing 3' splice acceptor motifs of HIV-1<sub>NL4.3</sub>. Mutations that ablated constitutive splice sites led to the activation of new cryptic sites; some of these preserved biological function. Mutations that ablated competing splice acceptor sites caused marked alterations in the pool of virus-derived mRNAs and, in some instances, in virus infectivity and/or the profile of virus proteins. The redundant RNA splicing signals in the HIV-1 genome and alternatively spliced mRNAs provides a mechanism for regulating the relative proportions of HIV-1 proteins and, in some cases, viral infectivity.

Eucaryotic cells control their metabolic activities by regulating gene promoter activity and the processing of RNA and protein. In the same way that the study of viral promoters has served as a paradigm for the promoters of their host mammalian cells, the investigation of viral RNA processing in mammalian cells has provided an insight into various mechanisms used to regulate the steady-state level of a specific mRNA. The complex nature of the processing of human immunodeficiency virus type 1 (HIV-1) RNAs provides an important model for human RNA processing pathways. All retroviruses require RNA splicing to remove upstream *gag* and *pol* coding sequences from the *env* mRNA. In addition, HIV-1 generates a distinctly complex pattern of spliced RNA to encode the essential regulatory proteins, Tat and Rev, as well as several other proteins (Vif, Vpr, and Nef) needed for successful replication *in vivo* (3, 15, 18, 26, 32, 35, 36, 43, 47, 52). The HIV-1 Rev protein binds viral RNA species that contain the Rev-responsive element (RRE), located in the *env* gene, thereby promoting the export, and possibly the stability and translation, of partially spliced and unspliced RNAs from the nucleus into the cytoplasm for its translation and/or packaging into progeny virions (2, 6, 7, 9, 12-14, 19, 20, 22, 23, 28, 33, 36, 38). The Rev-RRE system alleviates the paradoxical requirement for both spliced and unspliced HIV-1 RNA for successful virus replication. Rev protein also regulates the temporal change from multiply spliced HIV-1 RNAs to partially spliced or unspliced RNAs during productive virus infection (27, 29).

The splicing of HIV-1 RNA is extremely complex because of the presence of both constitutive and alternatively used 5' RNA splice donor (SD) and 3' splice acceptor (SA) motifs. Numerous weak SA motifs, located toward the center of the

genomic RNA, are competing points of ligation for splicing, and their alternate selection usually determines the protein encoded by the mature RNA (3, 15, 18, 35, 43, 47). However, some of these mRNAs are multicistronic, encoding more than one protein (15, 48, 49). Increased diversity of spliced mRNAs for several HIV-1 proteins results from the alternative cassetting of two noncoding exons into a proportion of transcripts (15, 43, 47). In addition, the use of several cryptic SA and SD sites may lead to the synthesis of novel chimeric viral proteins (5, 15, 46, 47). The varied use of these diverse splicing signals results in the synthesis of several sets of structurally different RNAs that serve as alternative templates for the translation of the same protein, including the viral envelope, regulatory, and accessory proteins. Because this complex pattern of RNA expression is maintained among many HIV-1 isolates of diverse origins (42, 43, 50), it is likely that this complexity is critical for the successful completion of the HIV-1 infectious cycle and not simply an inherent redundancy in viral RNA processing.

The major advantage of examining regulated splicing of a self-replicating entity such as HIV-1 is that such investigations can use whole cells, rather than *in vitro* splicing extracts, and the biological consequences can be readily correlated with RNA and protein expression as well as with virus infectivity. While mixed groups of mRNAs encode most HIV-1 proteins, it is unclear whether different cell types use alternative splicing to regulate HIV-1 RNA expression. The principal cell types that are infected by HIV-1, CD4<sup>+</sup> lymphocytes and monocyte-derived macrophages, are known to alternatively splice RNA from some cellular genes, depending on the maturation or activation status of the cell (e.g., CD45 [54, 55]) or on the cell type (e.g., CD46 [44]). Given the wide sequence diversity of HIV-1 strains (37), it is likely that sequence differences will

\* Corresponding author.

affect the splicing motifs of different virus isolates in view of what is known in other systems (17, 34).

We exploited the self-replicating capacity of HIV-1 to examine the role of alternative RNA splicing in the regulation of virus replication and infectivity and to evaluate the relative importance of the different RNAs encoding HIV-1 proteins. First, we used a semiquantitative polymerase chain reaction (PCR) protocol that preserved the relative proportions of the HIV-1 RNA species in the 1.8- or 4.0-kb class of poly(A)<sup>+</sup> RNA to evaluate the steady-state levels of viral mRNAs during productive viral infection. Second, we introduced mutations into several SD and SA motifs of the HIV-1 proviral clone pNL4-3 to assess their effects on the composition and relative abundance of alternatively spliced mRNAs during virus replication and infection. HIV-1 splice site mutants permitted an examination of the biological significance of the large redundant pool of spliced mRNAs and the potential role of alternative RNA splicing in the regulation of HIV-1 during tissue culture infections.

## MATERIALS AND METHODS

**Construction of proviral mutants.** The HIV-1 proviral molecular clone pNL4-3 was constructed from the NY5 and LAI (LAI) HIV-1 isolates (1). A PCR-based mutagenesis protocol that used a mutagenic oligonucleotide and a second primer positioned near a convenient restriction endonuclease site was used to generate a PCR product containing the mutation. This product was gel-purified and used as a megaprimer with a third oligonucleotide positioned near a second convenient restriction site to generate DNA containing the mutated splice site and the two restriction sites (31). These products were cloned into the pCR1000 vector (Invitrogen, San Diego, Calif.), screened by restriction mapping, and then cloned back into the HIV-1 provirus by using the selected restriction sites. The oligonucleotides used, with mutated nucleotides underlined, were as follows: for SD1, Odp.008 (5'-TGGCGTACTCTGC AGTCGCGGCC-3') with Odp.002 (5'-CTCTGGTAACTAG AGATCCCTCAG-3') and then Odp.007 (5'-CTCATCTGG CTGGTGCAATAAGG-3'); for SA4b, Odp.023 (5'-AGGAG ATGCTCAAGGCTTTGTCATG-3'), and for SA5, Odp.025 (5'-GTCTCCGCTTCTTCGAGGCCATAGG-3'), each with Odp.021 (5'-GATTGGGTGTCCGACATAGCAG-3') and then Odp.030, (5'-TTGTTTATTATTTTCCAAAATTGTCTC-3'); for SA6, Odp.028 (5'-GTGTTAGTTTATCTTC CACTGATTTTGAAG-3') with Odp.030 and then Odp.021; and for SA7a, Odp.033 (5'-CTATAGTGAATTCAGGTAG GCAGGGAT-3'), for SA7a+7b, Odp.035 (5'-CTATAGTGAATTCAGGTAG GCAGGGAT-3'), and for SA7a, 7b, Odp.037 (5'-CTATAGTGAATTCAGGTAG GCAGGGAT-3') with Odp.032 (5'-CCGAGATCGTCCCAGATAAG-3') and then Odp.031 (5'-AGTAGAGCAAAATGGATGCCAC-3'). Splice site mutant proviruses were sequenced to verify the presence of the desired change as well as additional changes that might have been introduced during the PCR procedure. Some mutations ( $\Delta$ SA4b Tat G→S,  $\Delta$ SA5 Tat R→S,  $\Delta$ SA6 Env K→S,  $\Delta$ SA7a Env R→S,  $\Delta$ SA7b Env R→S, and  $\Delta$ SA7 Env Q→R) changed the codon at that splice site. Other changes were as follows: pNL $\Delta$ SD1, 756 A→T (a silent change in the packaging signal); pNL $\Delta$ SA4b, 6002 C→T (Tat A→V, Rev L→F); pNL $\Delta$ SA5, 6143 A→G (Env E→G); pNL $\Delta$ SA6, 6695 T→C (Env F→L); and pNL $\Delta$ SA7a+7b, 7361 T→C (Env F→L), 8069 C→A (Env L→M), 8107 G→T (Env W→C), and 8321 T→C (Env S→P). Several other proviral splice site

mutants were sought; the resultant plasmids proved to be unstable, however, precluding their functional analysis.

**Cell culture, transfections, and infections.** HeLa cells, maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), were obtained from the American Type Culture Collection. CEM (12D7) cells, maintained in RPMI 1640 medium with 10% FCS, were obtained from Microbiological Associates (Gaithersburg, Md.), as were peripheral blood mononuclear cells (PBMC), which were stimulated with phytohemagglutinin (0.25 µg/ml; Wellcome Diagnostics, Dartford, United Kingdom) for 96 h and grown in RPMI 1640 with 10% FCS and 10% interleukin-2 (Pharmacia Diagnostics, Fairfield, N.J.). HeLa cells ( $5 \times 10^5$ ) in T25 flasks were cotransfected by the calcium phosphate coprecipitation technique (57) with 20 µg of proviral plasmid DNA and 0.5 µg of a human growth hormone reporter plasmid, pXGH5 (10); transfection efficiency was determined by a radioimmunoassay for human growth hormone (Nichols Institute, San Juan Capistrano, Calif.). Virus production was monitored with an assay for reverse transcriptase (RT) activity, using [<sup>32</sup>P]TTP incorporation with an oligo(dT)·poly(A) template (59). Cells ( $2 \times 10^5$ ) were infected with  $10^5$  cpm of RT activity of an HIV-1 inoculum (approximately equivalent to a multiplicity of infection of 0.002) in 1 ml of RPMI 1640 for 2 h at 37°C before addition of 4 ml of RPMI 1640 containing 10% FCS. The cells were fed with RPMI 1640 containing 10% FCS at 2-day intervals, and aliquots of the medium were assayed for RT activity.

**Isolation of HIV-1 mRNA, preparation of cDNA, and semiquantitative PCR for spliced HIV-1 mRNA.** Total cell RNA was harvested by extraction with RNeazol (TelTest Inc., Friendswood, Tex.) 16 or 36 h after transfection of HeLa cells or immediately prior to the peak of RT production following infection of approximately  $5 \times 10^6$  infected human PBMC; poly(A)<sup>+</sup> RNA was selected by the Micro-Fast track oligo(dT)-cellulose method (Invitrogen). For first-strand cDNA synthesis, either poly(A)<sup>+</sup> RNA or in vitro-transcribed RNA (see below) was denatured in 15 mM MeHgOH in the presence of 130 mM  $\beta$ -mercaptoethanol and 0.5 µg of random hexamers at 94°C for 2 min before reverse transcription (two times) for 1 h with murine leukemia virus RT, using a cDNA cycle kit (Invitrogen). Semiquantitative PCR analysis of cDNA from the 1.8-kb class of RNA was performed with oligonucleotide primers Odp.045 (5'-CTGAGCCGGAGCTCTCTGC3' positions 477 to 499) and Odp.032 (5'-CCGCAGATCGTCCCAGATAAG-3' positions 8477 to 8498); for the 4.0-kb class of RNA, primers Odp.045 and Odp.070 (5'-ACTATGTCTATTATTTATGTCTACTAC-3' positions 6094 to 6115) were used. Twenty cycles of PCR were performed with 1 U of AmpliTaq (Perkin Elmer Cetus, Norwalk, Conn.), 2 µl of first-strand cDNA, 0.2 mM each dATP, dCTP, dGTP, and dTTP, and 1 µM each primer in 10 mM Tris-HCl (pH 8.3)-50 mM KCl-1.5 mM MgCl<sub>2</sub>-0.001% (wt/vol) gelatin by first incubating the mixtures for 5 min at 94°C and then subjecting them to thermocycling (94°C for 1.5 min, 55°C for 1 min, 72°C for 2.5 min) before finally incubating them at 72°C for 7 min. If the DNA concentration in a reaction mixture was less than 10 ng/µl, as assessed by agarose gel electrophoresis, aliquots were sequentially reamplified in steps of five cycles after dilution 1:4 with fresh reaction mix to maintain linear amplification. Amplification products (100 ng) were radiolabeled by performing a single round of PCR as before but with addition of 10 µCi of [<sup>32</sup>P]dCTP and subsequently analyzed by electrophoresis on a 6% polyacrylamide-urea gel at sufficient current to maintain a temperature of 65°C; bands were visualized by autoradiography or quantified with a Fujix BAS 2000

Bio-image analyzer. An *Msp*I digest of pBR322 was end labeled and used as size markers. Controls used in PCR experiments included cDNA from mock-transfected and -infected cells and poly(A)<sup>+</sup> or in vitro-transcribed RNA (below) that was not incubated with RT during the cDNA synthesis reaction.

**Analysis of PCR products and cloning of HIV-1 cDNAs.** Bands were excised from the PCR gel, eluted in 0.5 M ammonium acetate-10 mM magnesium acetate-1 mM EDTA-0.1% sodium dodecyl sulfate (SDS) for 16 h at 4°C, and then precipitated with ethanol before reamplification with the same primers. After it was confirmed that the PCR product migrated as a single band, residual primers were removed with a Magic PCR Prep column (Promega, Madison, Wis.), and the product was directly sequenced by using end-labeled nested oligonucleotide primers in the dsDNA Cycle Sequencing System (Life Technologies, Inc., Gaithersburg, Md.). Purified PCR bands were also cloned into the pCRII vector (Invitrogen) so that the sense strand was downstream of the T7 promoter sequence, and the identities of clones were confirmed by sequencing. HIV-1 cDNA clones were linearized with *Spe*I, extracted with phenol and chloroform (50%, vol/vol), and precipitated with ethanol, and RNA was transcribed in vitro by using T7 RNA polymerase (Promega). The DNA template was removed by digestion (twice) with RNase-free DNase (Promega), and the RNA produced was directly quantified by spectrophotometry or density scanning of bands following agarose gel electrophoresis. Selected in vitro-transcribed RNAs were diluted and then mixed in known proportion as controls for subsequent cDNA synthesis and PCR amplification.

**Analysis of HIV-1 proteins.** Lysates were prepared from transfected HeLa cells ( $5 \times 10^7$  cells per ml) in 0.5% Nonidet P-40 in 10 mM Tris-HCl (pH 7.4)-150 mM NaCl-1 mM EDTA-1 mM phenylmethylsulfonyl fluoride. Different amounts of the cell lysate, standardized on the basis of human growth hormone activity, were separated on SDS-5 to 20% polyacrylamide gradient gels, electroblotted onto Immobilon-P membranes (Millipore, Bedford, Mass.), and blocked with 5% powdered skim milk in phosphate-buffered saline. HIV-1 proteins were detected by using serum (1:1,000) from an HIV-1-seropositive individual, or rabbit serum to a Nef N-terminal peptide (1:100) (21) or to purified NL4-3 gp160/120 (1:100) (58), and visualized with <sup>125</sup>I-labeled protein A by autoradiography or phosphoimaging analysis. Alternatively, cells were biosynthetically labeled with [<sup>35</sup>S]methionine-cysteine (Trans<sup>35</sup>S-label; ICN, Irvine, Calif.) as described previously (59) and immunoprecipitated with rabbit serum to Rev produced in *Escherichia coli* (1:100) (gift from D. M. D'Agostino and G. N. Pavlakis). Levels of Rev functional activity were measured as described previously (24) by cotransfected 0.5 µg of the pDM128 Rev reporter plasmid with 20 µg of proviral plasmid and 0.5 µg of pXGH5 human growth hormone reporter plasmid and then assaying for chloramphenicol acetyltransferase (CAT) activity on cell extracts that were standardized for growth hormone expression.

## RESULTS

The selection of different competing SA sites in primary HIV-1 transcripts leads to the alternative exon composition of fully processed mRNA (Fig. 1). For example, functional Tat protein, a transactivator of HIV-1 transcription (8, 35, 40, 51), is expressed from the two types of mRNA using SA4 (Fig. 1A): RNAs containing exon 4E, which is continuous from SA4 to the poly(A) addition site (one-exon *tat*), or transcripts joining exons 4 and 7 (two-exon *tat*) (Fig. 1C) (45). Two other

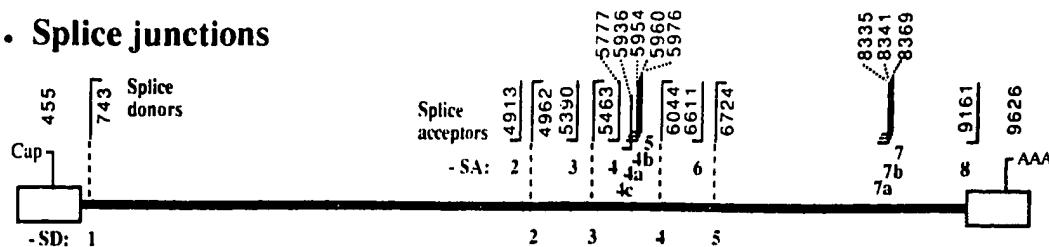
competing SA sites, SA4a and SA4b (Fig. 1A), mapping fewer than 200 bases downstream from SA4, give rise to exons 4a and 4b, which are spliced to exon 7 to generate mRNA for Rev (Fig. 1C). Another competing splice site, SA5 (Fig. 1A), is used for the expression of exons 5 and 5E (Fig. 1C); RNA species that contain exon 5E encode envelope gp160, and those splicing exon 5 to exon 7 encode Nef protein (15, 47). The translation initiation sites of mRNAs using SA4a, SA4b, and SA5 have poor ribosome binding capacities and therefore have the potential to be multicistronic: mRNAs containing exon 5E, 4aE, or 4bE encode Vpu and gp160 envelope proteins, whereas transcripts joining exon 4a or 4b with exon 7 encode both Rev and Nef (15, 47, 48, 53). This increases the coding potential of several HIV-1 mRNAs and the complexity of HIV-1 mRNA pool. Further complexity results from the interchangeable incorporation of two noncoding exons, exons 2 and 3 (Fig. 1C), into the spliced RNA species utilizing any of the downstream SA motifs.

PCR protocols using primers that promote amplification of limited subgroups of RNA have been used in conjunction with selective hybridization probes to map SA6, SD5, SA7a, and SA7b (Fig. 1A) within *env*, thus generating frameshifting exons 6, 7a, and 7b (Fig. 1C). These exons may generate novel chimeric Tat-Env-Rev (Tev or Tnv), Rev-X-Tat, and Tat-Env fusion proteins following transfection-infection by some derivatives of the HIV-1<sub>AI</sub> strain (5, 15, 46, 47). These are merely cryptic sites in the HIV-1<sub>NL4-3</sub> genome (see below), and their role during HIV-1 replication is not clear (16).

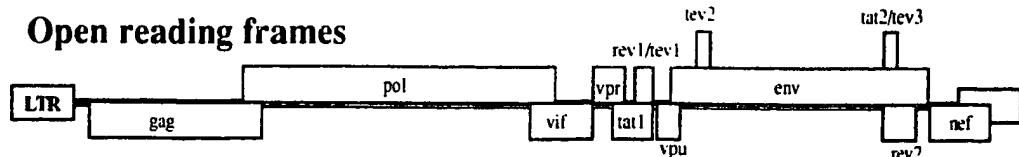
## Semiquantitative PCR analysis of spliced HIV-1 mRNA

Since Northern (RNA) blot analysis of HIV-1 RNA does not distinguish the full array of alternatively spliced RNAs encoding the same viral protein, we used a sensitive semiquantitative PCR assay and urea-acrylamide gel analysis that discriminated between RNA species differing in size by a single nucleotide. Two such assays were carried out. The first selectively detected the smaller multiply spliced 1.8-kb RNAs that use SD4 and SA7, SA7a, or SA7b (*nef*, *rev*, *tat*, or *vpu*) by using the Odp.045 and Odp.032 primers (Fig. 1D); the second specifically analyzed the larger 4.0-kb RNA species, which contain exons that extend beyond SD4 into the *env* reading frame (*vpu/env*, one-exon *tat*, *vpr*, and *vif*), by using the Odp.045 and Odp.070 primers (Fig. 1E). Representative gels, depicting the PCR products that resulted from random hexamer priming of the multiple species of HIV-1 RNA present in infected PBMC prior to the peak of RT production, are shown in Fig. 1D and E. Each of these cDNA bands was excised from the gel, reamplified, and directly sequenced. Several representative cDNA clones of each band were also introduced into the pCRII vector, which contains a T7 promoter adjacent to the cloning site, and also sequenced. To ascertain whether the relative quantity of the PCR-amplified cDNA bands visualized on the gel faithfully represented the relative levels of the various RNA species isolated from infected cells, plus-sense HIV-1 RNA was synthesized from eight different cDNA clones in vitro by using T7 RNA polymerase, directly quantified, and then mixed in known proportions. Included among these RNAs was one, designated cryptic, from a cDNA clone containing exons 1, 2x, 5, and 7, where exon 2x reads through SD2 to a cryptic SD at position 5059 in *vif*. The RNA mixtures were then used as templates for cDNA synthesis and PCR amplification, and the <sup>32</sup>P-labeled PCR products generated were analyzed by phosphoimaging (Fig. 2). We found that the proportion of radioactivity measured for each cDNA band closely matched the proportion of RNA added to the mixture prior to cDNA synthesis (Fig. 2A). Thus, each cDNA reverse

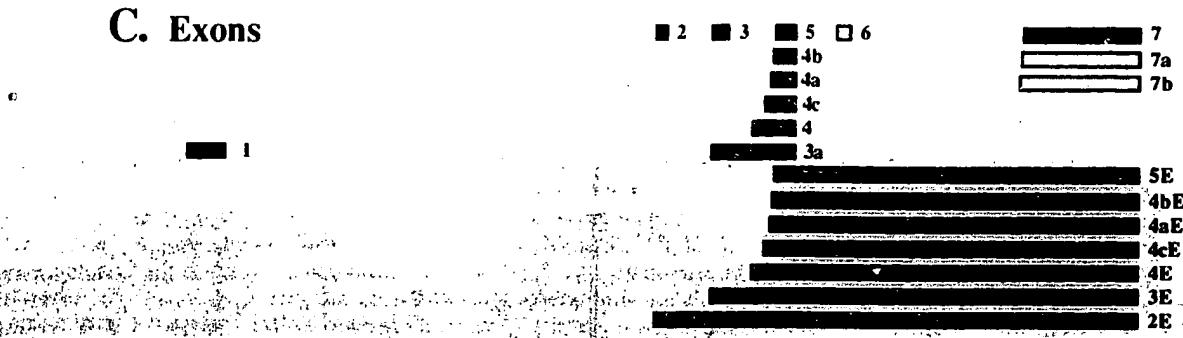
### A. Splice junctions



### B. Open reading frames

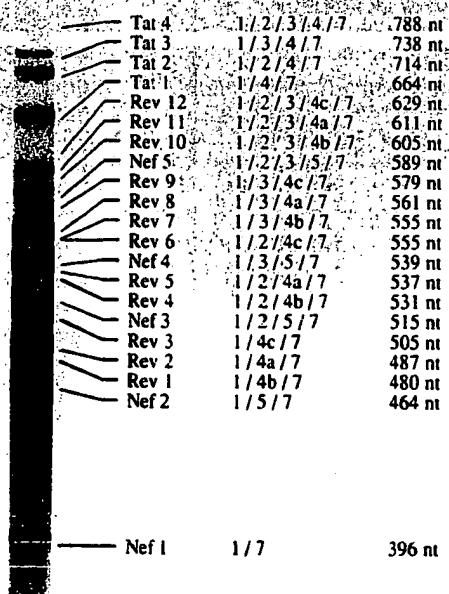


### C. Exons



### D. PCR for 1.8 kb class of RNA

PCR Gel	RNA name	Exon content	Band size
	Vpr 2	1/2/3a/7	1101 nt
	Vpr 1	1/3a/7	1051 nt



### E. PCR for 4.0 kb class of RNA

PCR Gel	RNA name	Exon content	Band size
	Vif 2	1/2E	1474 nt
	Vpr 4	1/2/3E	1047 nt
	Vpr 3	1/3E	997 nt
	Tat 8	1/2/3/4E	734 nt
	Tat 7	1/3/4E	684 nt
	Tat 6	1/2/4E	660 nt
	Tat 5	1/4E	610 nt
	Env 16	1/2/3/4cE	575 nt
	Env 15	1/2/3/4aE	557 nt
	Env 14	1/2/3/4bE	551 nt
	Env 13	1/2/3/5E	535 nt
	Env 12	1/3/4cE	525 nt
	Env 11	1/3/4aE	507 nt
	Env 10	1/3/4bE	501 nt
	Env 9	1/2/4cE	501 nt
	Env 8	1/3/5E	485 nt
	Env 7	1/2/4aE	483 nt
	Env 6	1/2/4bE	477 nt
	Env 5	1/2/5E	461 nt
	Env 4	1/4cE	451 nt
	Env 3	1/4aE	433 nt
	Env 2	1/4bE	426 nt
	Env 1	1/5E	410 nt

transcribed from the in vitro RNA was amplified with equal efficiency with use of the Odp.045-Odp.032 primer pair.

PCR analysis of the 1.8-kb class of mRNA was performed on samples from HeLa cells transfected with HIV-1 proviral clones or from human PBMC infected with cell-free HIV-1 (Fig. 2B). RNA directed by the pNL4-3 cloned provirus DNA in transfected HeLa cells or by HIV-1<sub>NL4-3</sub> in infected human T lymphocytes yielded the same pattern of bands in numerous independent experiments irrespective of whether RNA was synthesized following transfection or infection. A similar banding pattern was also observed with RNA prepared from PBMC infected with HIV-1<sub>LAI</sub> (Fig. 2B, lane 10). The results presented in Fig. 2 demonstrate that HIV-1 RNA is spliced equivalently in transfected HeLa cells and infected PBMC and that the splicing pattern is similar for closely related strains of HIV-1 such as NL4-3 and LAI.

These control PCR amplifications also illustrate two other important points: (i) for each RNA mixture, the relative proportion of the different amplified RNA species, as determined experimentally, closely approximated the original proportion added, and (ii) only PCR products deriving from input RNA were detected on the gel, indicating that only RNAs spliced by genuine processing pathways gave bands in this analysis. Abnormal cDNA species, potentially arising by template jumping during reverse transcription of RNA, did not appear as bands on the final autoradiogram. Thus, the PCR protocol used here accurately reflects the relative quantity of one HIV-1 RNA species compared with the others amplified in the same reaction.

**Identification of a new SA site for rev and vpu/env RNAs.** Our PCR analyses of viral RNA synthesized in transfected HeLa cells and infected PBMC indicated the presence of a previously unreported competing SA site, designated SA4c, that was used to generate three novel rev mRNAs, rev3, rev6, and rev9 (Fig. 1D), and three novel vpu/env mRNAs, env4, env9, and env16 (Fig. 1E). The SA4c site is 18 nucleotides (nt) upstream from SA4a and is conserved among most, but not all, HIV-1 isolates (Fig. 3). The SA4c splice site exists in HIV-1<sub>IIIB</sub>, HIV-1<sub>LAI</sub>, HIV-1<sub>JR-CSF</sub>, and HIV-1<sub>BA-L</sub>, the strains used in earlier studies mapping the splicing motifs of HIV-1, although its use was not noted (3, 15, 18, 35, 43, 47). Since no new translation initiation sites are introduced into mRNAs using SA4c, transcripts containing exons 4c and 4cE would still encode typical HIV-1 Rev or Env protein. However, structural changes introduced into these mRNAs may affect their translatability. An ATG codon exists in the HIV-1<sub>NL4-3</sub> between SA4c and SA4a but is present in a context unfavorable for efficient translation (30).

**Relative abundance of the alternatively spliced HIV-1 mRNAs.** Since PCR amplification of HIV-1 cDNA preserved the relative proportion of the various alternatively spliced forms of HIV-1 RNA, we used phosphoimaging analysis to directly determine the relative abundance of each of these RNAs in a spreading HIV-1<sub>NL4-3</sub> infection of PBMC (Fig. 2 and 4). Within the 1.8-kb class of HIV-1 mRNAs (Fig. 4A), nef, rev, tat, and vpr species existed in a ratio of 56:34:9:1; within the 4.0-kb class (Fig. 4B), env, tat, vpr, and vif species existed in a ratio of 92:5:2:1. Of the nef mRNAs, nef2, which includes noncoding exon 5 flanked by SA5 and SD4, was the predominant type, comprising 49% of all nef RNA and 28% of all 1.8-kb RNAs. nef RNAs containing noncoding exon 3 (nef4) or 2 (nef3) or both 2 and 3 (nef5) were present in decreasing amounts. Nef1 RNA, which lacks a noncoding exon, was the least abundant nef mRNA. It is unclear whether the nontranslated RNA from exon 5 contributes to nef function, as the nef gene has not been evaluated as an RNA element, only as protein.

In contrast, the predominant spliced mRNAs for the other HIV-1 proteins lacked noncoding exons. Only 20% of rev mRNA includes a noncoding exon, and the use of noncoding exon 3 (rev7, rev8, and rev9) or both exons 2 and 3 (rev10, rev11, and rev12) predominates over use of exon 2 alone (rev4, rev5, and rev6). In addition, rev1 and rev2 mRNAs, utilizing SA4a and SA4b, respectively, occur approximately fivefold more frequently than rev3 mRNA, which uses the previously unreported SA4c. Both the one- and two-coding-exon forms of tat mRNA (1.8- and 4.0-kb class RNAs, respectively) infrequently use noncoding exons 2 and 3 (tat1 and tat5). However, when a tat mRNA contained a noncoding exon, exon 2 was predominantly used (tat2 and tat6). The relative proportion of the one- and two-exon forms of tat or vpr mRNA could not be determined here, since the assays for the 1.8- and 4.0-kb mRNAs are merely semiquantitative among the species represented in each PCR assay.

Eighty percent of env mRNAs use SA5 (exon 5E); however, 12% of env mRNAs (env2, env3, and env4) utilize the upstream SA4a, SA4b, or SA4c (exon 4aE, 4bE, or 4cE) when noncoding exons 2 and 3 were excluded. Noncoding exons 2 and 3 were used only at low (<5%) frequency in env mRNA and mostly in conjunction with SA5 (env5, env8, and env13). This study clearly identifies mRNA species containing both noncoding exons 2 and 3 in the same transcript. This finding contrasts with previous analyses of RNA directed by the HIV-1<sub>IIIB</sub> strain that identified these as mutually exclusive exons (15, 18, 47) but is in agreement with a recent report that characterized viral

**FIG. 1.** Identification by RT-PCR of alternatively spliced HIV-1 mRNAs containing a variety of exons that arise from the existence of numerous splice junctions encoded in the HIV-1 genome. (A) Map showing the locations of the SD and SA sites in the pNL4-3 proviral molecular clone of HIV-1, with each position shown in nucleotides from the start of the 5' long terminal repeat (LTR). The SD and SA sites are numbered as done by Schwartz et al. (47). (B) Organization of the HIV-1 genome. Open boxes show locations of the open reading frames that encode the HIV proteins. (C) The different HIV-1 exons generated from the use of different combinations of SA and SD motifs during RNA splicing are shown as bars and numbered as done by Muesing et al. (35). Exons represented by gray bars were not found in any of the RNA species examined in these studies. Exons 1, 2, 3, and 5 do not encode HIV-1 protein, and those numbered with an E read through SD4 into the env gene. (D) The various HIV-1 mRNA species falling into the 1.8-kb size range in Northern blot analyses were distinguished by acrylamide gel analysis of PCR-amplified cDNA, using primers Odp.045 (positions 477 to 499) and Odp.032 (positions 8477 to 8498). A representative gel from PCR analysis of RNA from pNL4-3-infected PBMC is shown. The distinct HIV-1 RNA species were identified by direct PCR sequencing of excised bands and by cloning, and the identity, exon content, and size of the PCR product are shown. The mRNA species have been named according to the principal protein that they encode and by their size, with the smallest RNA as 1. Faint bands were visible on longer exposures of the autoradiogram. rev mRNAs are bicistronic and also encode Nef (15, 48, 49). (E) Representative acrylamide gel from PCR analysis of HIV-1 mRNA species falling into the 4.0-kb size range from pNL4-3-infected PBMC, using primers Odp.045 and Odp.070 (positions 6194 to 6115). The identity of each RNA species, determined by direct sequence analysis of excised bands, is shown with the exon content and size of the PCR product. RNA species not matched to a band on the gel were difficult to detect except on very long gel exposures and were not quantified above the background level in phosphoimaging analysis. All env mRNAs are multicistronic and also encode Vpu and Nef (48, 49).

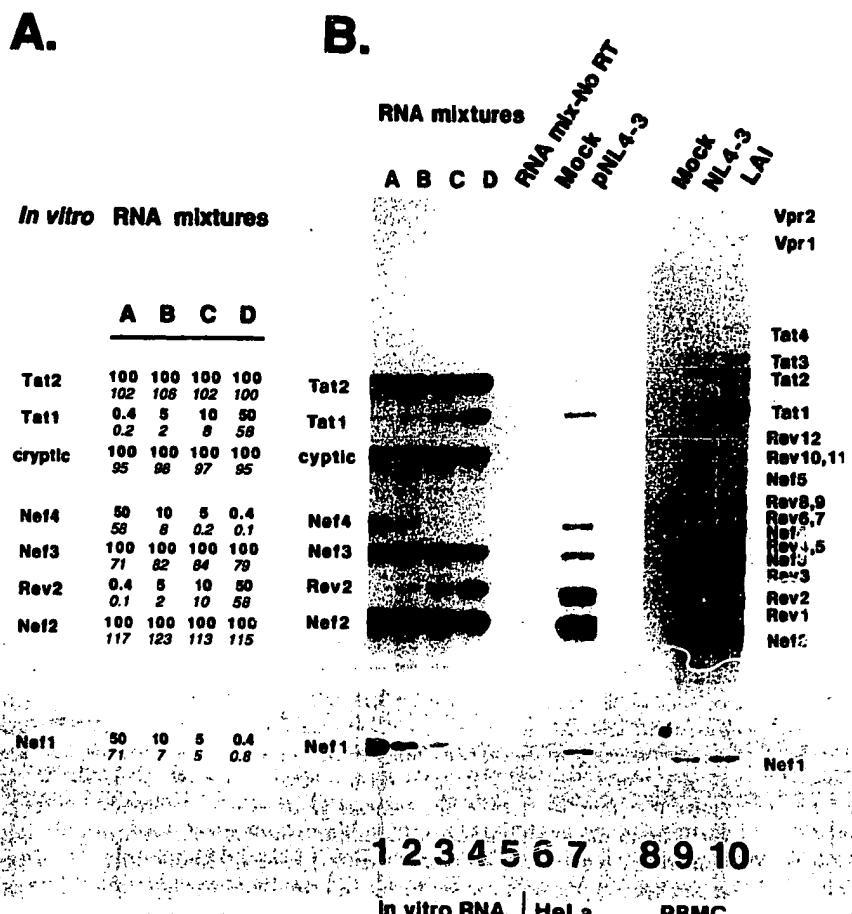


FIG. 2. The relative proportions of alternatively spliced HIV-1 mRNAs were accurately determined by RT-PCR assay. The semiquantitative capacity of the RT-PCR assay for the 1.8-kb class of spliced HIV-1 mRNA was evaluated by mixing predetermined concentrations of RNA transcribed in vitro from HIV-1 cDNA clones with T7 RNA polymerase (A) and then performing reverse transcription, PCR amplification, labeling with [ $^{32}$ P]dCTP, and gel analysis (B). Proportions of each RNA added into four mixtures (A through D) are shown in panel (A) with the proportion of cDNA experimentally amplified by PCR (panel B, lanes 1 to 4) and determined by phosphoimaging analysis (shown in italics in panel A). (B) Lane 5 shows a pool of RNA mixtures A through D that were treated in parallel but without the addition of RT during cDNA synthesis. RNA from HeLa cells transfected with reporter-human growth hormone only (lane 6) and with pNL4-3 (lane 7) or with RNA from PBMC (lane 8) and PBMC infected with HIV-1NL4-3 (lane 9) or HIV-1LAI (lane 10) was examined in the same set of reactions as the control. The identity of each amplified species is shown on the right (see Fig. 1D).

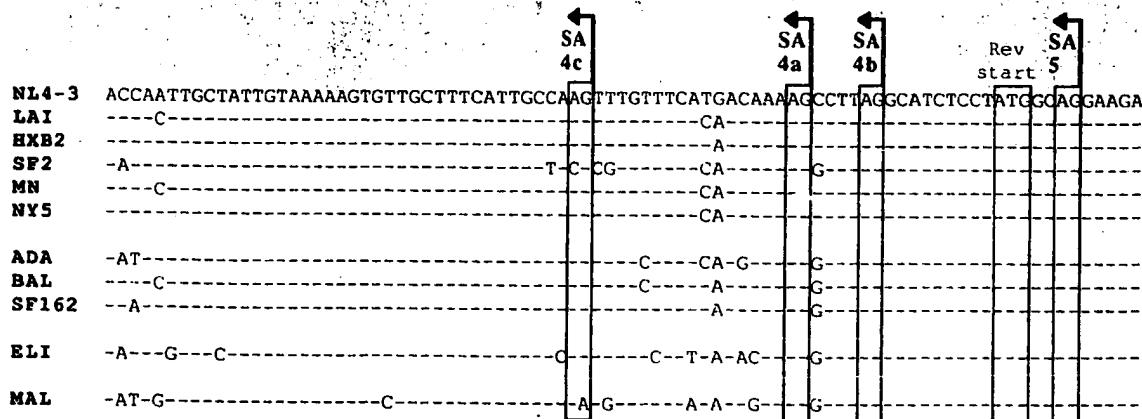


FIG. 3. Location of SA4c, a new SA site for *rev* and *vpu/env* mRNAs. Shown is alignment of nucleotide sequences of several HIV-1 isolates surrounding the new SA site for *rev* and *vpu/env* mRNAs. SA4c (adapted from Myers et al. [37]).

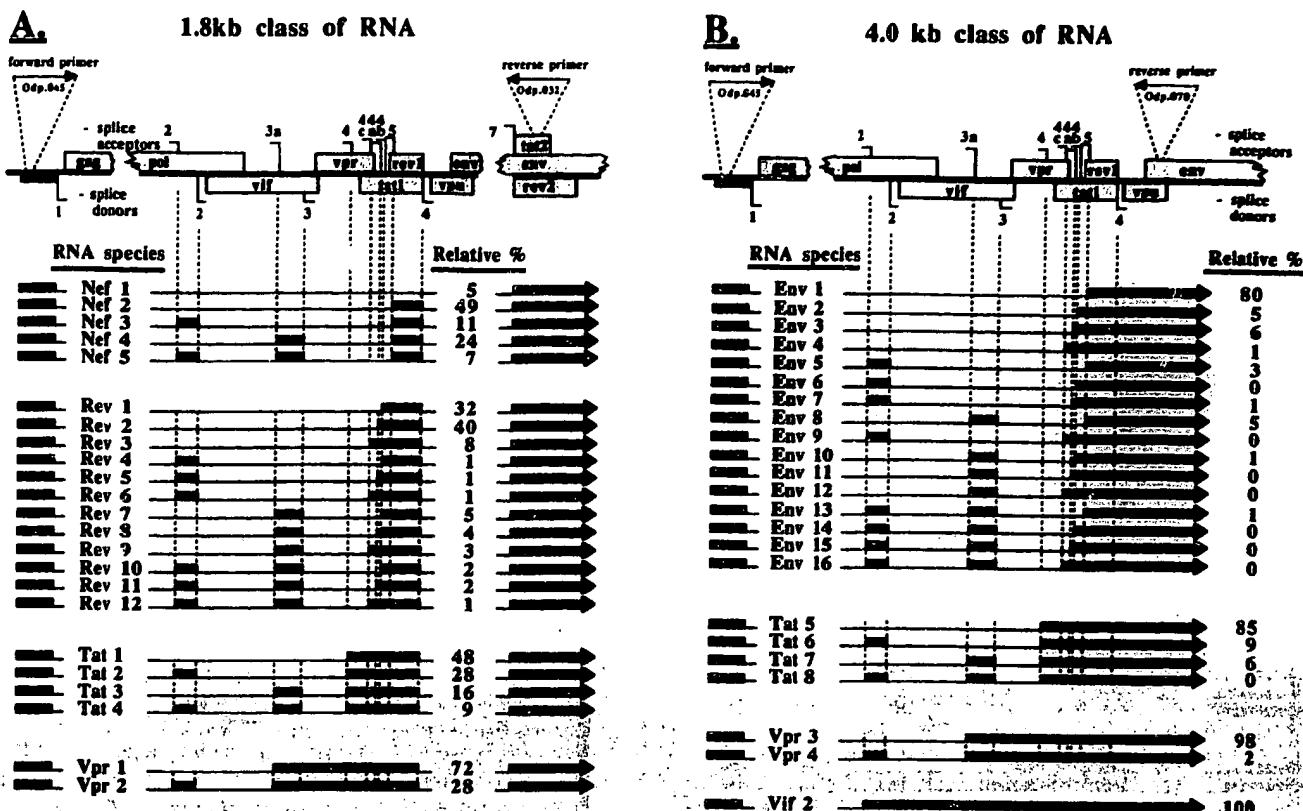


FIG. 4. Structure and relative abundance of each alternatively spliced HIV-1 mRNA. (A) Splice site usage of the 1.8-kb class of HIV-1 mRNA amplified by PCR of randomly primed cDNA, using primers Odp.045 and Odp.032. (B) Splice site usage of the 4.0-kb class of HIV-1 mRNA amplified by PCR of randomly primed cDNA, using primers Odp.045 and Odp.070. The solid boxes raised above the line represent the regions of retained RNA. Shown at the right of each panel is the relative proportion of each mRNA species quantified by phosphoimaging from pNL4-3 virus infections of PBMC, using semiquantitative PCR analysis, for the 1.8- and 4.0-kb classes of RNA. In general, values less than 1% were not measured at levels significantly above background and were recorded as zero; however, the existence of these cDNA species was evident on long gel exposures.

RNA in infected MT-2 cells (50). No evidence was found for a *vif* RNA among the 1.8-kb species that spliced SD4 to SA7 (vif1).

**Splice site mutants of pNL4-3.** During HIV-1 mRNA splicing, the cellular spliceosome cleaves at GT and AG dinucleotides within the SD and SA motifs, respectively. Several of these highly conserved dinucleotide motifs present in the pNL4-3 proviral DNA clone of HIV-1 were replaced with different dinucleotides to block RNA splicing, using a PCR-based strategy (Fig. 5). Seven site-directed provirus mutants were generated by inactivating (i) the constitutively used major splice donor, pNL $\Delta$ SD1; (ii) the competing splice acceptor for the first coding exon of *rev*, pNL $\Delta$ SA4b; (iii) the competitively selected major splice acceptor for *env* and *vpu*, pNL $\Delta$ SA5; (iv) the cryptic splice acceptor within *env* purportedly used to generate the Tev protein (5, 46, 47), pNL $\Delta$ SA6; (v) SA7a, the most 5' of two conserved cryptic SA sequences mapping 34 and 28 bases upstream from the second coding exon of *tat* and *rev*, designated pNL $\Delta$ SA7a; (vi) both the SA7a and SA7b cryptic sites, pNL $\Delta$ SA7a+7b; or (vii) both of these in combination with SA7, the constitutive SA for the second coding exon of *tat* and *rev*, pNL $\Delta$ SA7+7a+7b. Some of these mutations also altered an amino acid codon(s) overlapping the splice site (Materials and Methods). The integrity of all mutant proviral clones was confirmed by nucleotide sequence analysis of the reconstructed regions.

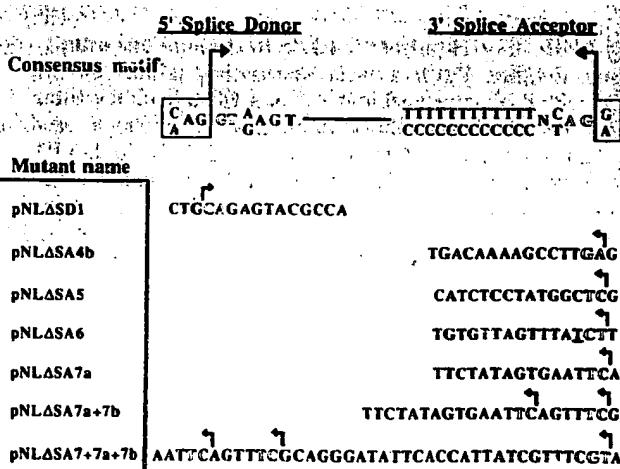


FIG. 5. Base substitutions introduced into SD and SA motifs of the pNL4-3 proviral clone of HIV-1. The consensus motifs of mammalian SD and SA sites are shown at the top, with the essential two dinucleotides of the motif shown in outlined font. These dinucleotides were changed in the pNL4-3 proviral clone to the bases shown in outlined font so as to inactivate several SD and SA sites. Arrows indicate the point of cleavage and ligation of RNA during splicing.

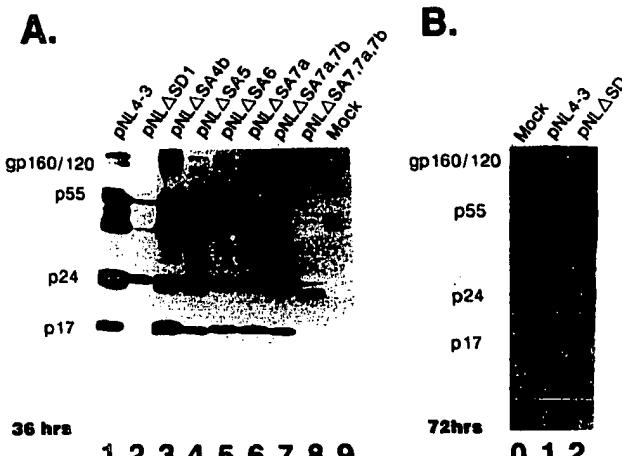


FIG. 6. Some HIV-1 splicing mutants direct the synthesis of an altered profile of HIV-1 protein. Western blot analysis of HIV-1 protein detected with patient serum from HeLa cell lysates was performed 48 h (A) and 72 h (B) after cotransfection of 0.5  $\mu$ g of a growth hormone reporter plasmid with 20  $\mu$ g of wild-type pNL4-3 (lane 1), with RNA splice mutant provirus pNLΔSD1 (lane 2), pNLΔSA4b (lane 3), pNLΔSA5 (lane 4), pNLΔSA6 (lane 5), pNLΔSA7a (lane 6), pNLΔSA7a+7b (lane 7), or pNLΔSA7+7a+7b (lane 8), or with reporter plasmid alone (lanes 9 and 0). The volume of cell lysate was standardized for transfection efficiency according to the determination of human growth hormone in the culture supernatants.

**Protein synthesis by splicing mutants of HIV-1:** In the first group of experiments, the effects of the splice site mutations on viral protein production were assessed by Western blotting (immunoblotting) lysates from transfected HeLa cells, using an AIDS patient's serum. As shown in Fig. 6, each of the mutants except pNLΔSA7+7a+7b directed the synthesis of the same complement of HIV-1 proteins as did wild-type pNL4-3, with the following exceptions. Mutation of SD1 caused a marked decrease in the quantity of viral proteins accumulating in HeLa cells during the first 48 h despite transfection efficiency equivalent to that of the wild type, as measured by coexpression of a human growth hormone expression plasmid (Fig. 6A, lanes 1 and 2). By 72 h after transfection, however, HIV-1 protein accumulation directed by mutant plasmid pNLΔSD1 was comparable to the wild-type level (Fig. 6B, lanes 1 and 2). Mutation of SA4b, used for the production of *rev* and *env* mRNAs, led to consistent and substantial elevations of gp160/120 levels compared with the wild type (Fig. 6A, lane 3). In contrast, the mutation of SA5 markedly reduced, but did not eliminate, gp160/120 production (Fig. 6A, lane 4). Mutation of SA6 (for *rev* mRNA) and the SA7a or SA7a+7b cryptic splice sites (for the second coding exon of *tat* and *rev*) had little if any effect on HIV-1 protein synthesis (Fig. 6A, lanes 5 to 7), indicating that these three SA sites (and exons 6, 7a, and 7b) play no significant role for HIV-1 protein synthesis in transfected HeLa cells. In contrast, mutant pNLΔSA7+7a+7b, which contains a triple splice site mutation including SA7, the in-frame acceptor used constitutively for the second coding exon of *tat* and *rev*, directs the synthesis of a markedly altered protein profile (Fig. 6A, lane 8), similar to that previously reported for Rev-deficient HIV-1 mutants: minimal levels of p55, p24, or p17<sup>\*\*\*</sup> protein and no detectable gp160/120 (12, 14, 20, 33) accompanied by a novel and abundant 20-kDa protein that reacted with Tat antiserum (data not shown). No increased accumulation of HIV-1 structural proteins was noted at later times (72 or 96 h after transfection) despite the

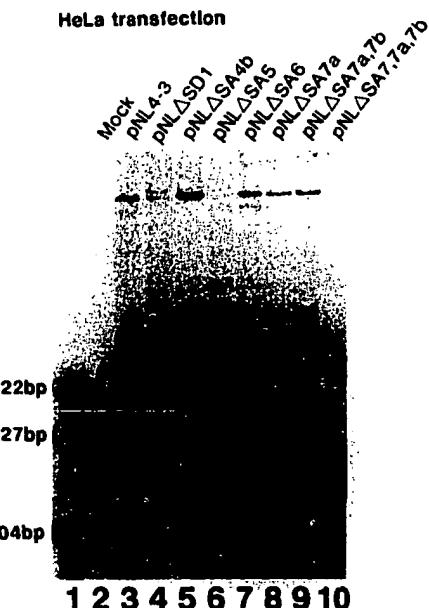


FIG. 7. HIV-1 proviruses mutated at cryptic splice sites synthesize a wild-type profile of RNA. Semiquantitative RT-PCR analysis of the 1.8-kb class HIV-1 RNA from HeLa cells transfected with 0.5  $\mu$ g of reporter plasmid alone (lane 2) or cotransfected with 20  $\mu$ g of pNL4-3 (lane 3), or HIV-1 RNA splicing mutant pNLΔSD1 (lane 4), pNLΔSA4b (lane 5), pNLΔSA5 (lane 6), pNLΔSA6 (lane 7), pNLΔSA7a (lane 8), pNLΔSA7a+7b (lane 9), or pNLΔSA7+7a+7b (lane 10). An *Msp*I digest of pBR322 is shown on the left (lane 1) as a size marker.

expression of levels of total cellular HIV-1 RNA similar to wild-type levels by Northern blot analysis (not shown). These results suggest that mutant pNLΔSA7+7a+7b failed to express functional Rev and resulted in reduced amounts of Rev-dependent cytoplasmic mRNAs encoding Gag and Env proteins.

**mRNA analysis from cryptic splice site mutants of HIV-1:** When the 1.8-kb class of RNA from HeLa cells transfected with HIV-1 proviral DNA clones containing splice site mutations was analyzed, it was clear that several expressed the same RNA pattern as did wild-type pNL4-3 (Fig. 7). For example, mutants pNLΔSA6, pNLΔSA7a, and pNLΔSA7a+7b were indistinguishable from the wild type (Fig. 7, lanes 7 to 9), indicating that the SA6, SA7a, and SA7b sites are not used with significant frequency by HIV-1<sub>NL4-3</sub>, even though it is virtually identical to HIV-1<sub>IXB2</sub>, the strain in which these splice sites were originally described. Furthermore, no cDNAs using the SA6, SA7a, or SA7b splice site were ever detected among the PCR-amplified bands that were directly sequenced from cells transfected or infected with derivatives of HIV<sub>LA1</sub> or HIV-1<sub>NL4-3</sub>, and no clone generated from these PCR-amplified cDNAs could be shown to utilize these splice sites. Thus, the SA6, SA7a, and SA7b cryptic sites are used extremely rarely, if at all, and may be active only in the HIV-1<sub>LA1</sub> derivative HIV-1<sub>IXB2</sub>.

We found that careful selection and testing of PCR primers was required, in conjunction with the use of poly(A) RNA and nonspecific cDNA priming, to avoid the amplification of aberrant cDNAs. Our semiquantitative PCR assays depended on equal use of all the HIV-1 RNA species in either the 1.8- or 4.0-kb mRNA classes as competing templates for amplification. Other protocols may selectively amplify transcripts using SA6, SA7a, and SA7b (47); reports characterizing the se-

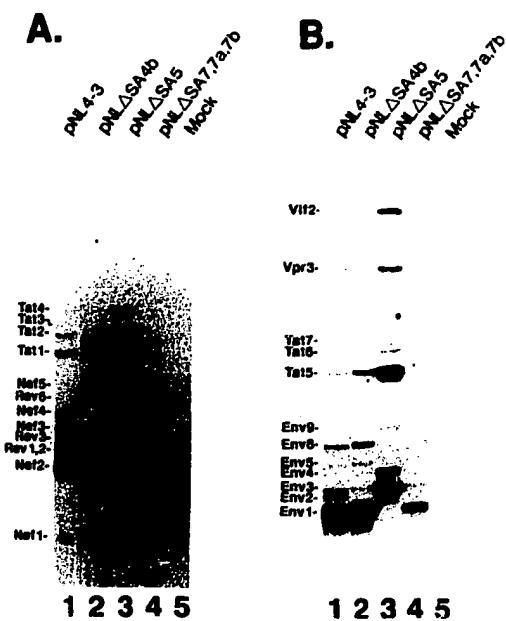


FIG. 8. An altered profile of spliced RNA is synthesized by HIV-1 provirus mutated at constitutive or competing splice sites. Shown is semiquantitative RT-PCR analysis of the 1.8-kb class HIV-1 RNA (A) and the 4.4- to 5.5-kb class of HIV-1 RNA (B) from HeLa cells transfected with 20  $\mu$ g of pNL4-3 (lane 1) or HIV-1 RNA splicing mutant pNL $\Delta$ SD1 (lane 2), pNL $\Delta$ SA4b (lane 3), pNL $\Delta$ SA5 (lane 4), or pNL $\Delta$ SA7+7a+7b (lane 5).

quence of cDNA clones around the SA7 splice site (15) or using hybridization analyses of cDNAs (50) also failed to detect usage of SA7a or SA7b.

**Analysis of mRNA from HIV-1 with mutated constitutive and competitive splice sites.** The cDNAs amplified from cells transfected with mutant pNL $\Delta$ SD1 had a pattern very similar to that of the wild type but with a slightly slower electrophoretic mobility (compare lanes 3 and 4 in Fig. 7). Direct sequence analysis of these PCR bands as well as several individual cDNA clones indicated that spliced RNA from pNL $\Delta$ SD1 used a GT dinucleotide 4 nt downstream from the mutated major SD (constitutive SD1) site as a cryptic SD site in this HIV-1 proviral DNA. The HIV-1 proteins translated from these slightly larger mRNAs were indistinguishable from the wild type (Fig. 6), but the rate of splicing of these viral transcripts may be slower, perhaps explaining the delay in protein expression previously observed. This cryptic SD (incorrectly annotated as the major SD in the alignment of this region by Myers et al. [37]) is strongly conserved among all HIV-1 isolates. Because the cryptic SD1 site may be used only when the genuine site is inactivated, it is likely that the strong conservation of this alternative splice site results from additional selective pressures. Nevertheless, its existence greatly reduces the possible loss of virus infectivity due to a spontaneous mutation affecting the major SD that would otherwise block the production of functional spliced mRNAs.

The cDNAs amplified from the remaining mutants differed from the pNL4-3 wild-type pattern (Fig. 7 and 8). For the provirus mutant inactivating the constitutive SA7 for the second coding exon of *tat* and *rev*, pNL $\Delta$ SA7+7a+7b, every cDNA band clearly differed in size compared with the wild type following amplification of the 1.8-kb species of HIV-1 mRNA (Fig. 8A, lane 4); the overall pattern was similar to the wild-type pattern except that each band had a faster electro-

phoretic mobility. A direct sequence analysis of these PCR bands and sequencing of individual pNL $\Delta$ SA7+7a+7b cDNA clones indicated that all used an AG dinucleotide situated 20 nt downstream from SA7 as the alternative SA site; this resulted in cDNAs that were 20 bases shorter than the wild type. The activation of this cryptic downstream SA site, however, precluded the generation of mRNA capable of encoding a functional Rev protein and resulted in an immunoblot devoid of HIV-1 structural proteins (Fig. 6A, lane 8). It should be noted that all of the wild-type 1.8-kb spliced RNAs were transcribed as truncated species by mutant pNL $\Delta$ SA7+7a+7b except for the nef1 RNA, which was not detected on the autoradiogram shown in Fig. 8A. The cDNA bands obtained for pNL $\Delta$ SA7+7a+7b after PCR for the 4.0-kb mRNA were identical to those of wild-type virus, indicating that RNA splicing to SA and SD sites upstream of SA7 was not affected by the absence of functional two-exon Rev protein. This finding demonstrates that Rev is not required for splice site selection in HIV-1 RNAs; however, protein expression from RNAs containing an RRE clearly requires functional Rev protein.

Mutation of each of two competing SA sites, SA4b and SA5, caused a different usage frequency for neighboring SA sites but not the activation of any cryptic sites. The first competing SA mutant, pNL $\Delta$ SA4b, failed to generate bands for rev1 (Fig. 8A, lane 2), rev4, rev7, and rev10 (evident after long gel exposures; not shown) following PCR with primers for the 1.8-kb mRNA species and for env2 (Fig. 8B, lane 2), env6, env10, and env14 (evident after long gel exposures; not shown) after PCR with primers for the 4.0-kb mRNA. The failure to detect these bands was consistent with the absence of RNAs splicing to the mutagenized SA4b site. All other cDNAs amplified from pNL $\Delta$ SA4b were identical to wild-type cDNAs. The cDNA pattern associated with the second competing SA mutant, pNL $\Delta$ SA5, lacked several predominant nef species (nef2, nef3, nef4, and nef5) after PCR for the 1.8-kb mRNA (Fig. 8A, lane 3), as well as the major env species (env1, env5, env8, and env13), after PCR for the 4.0-kb mRNA (Fig. 8B, lane 3), reflecting the absence of RNAs splicing to the mutagenized SA5 site. In addition, there was a compensatory increase in the use of SA4, SA4a, SA4b, and SA4c, resulting in increased levels of rev1, rev2, rev3, tat1, tat2, tat3, tat4, tat5, tat6, tat7, env2, env3, and env4 mRNAs.

**Translational consequences of altered RNA profile for competitive splice site mutants.** Since the mRNAs using the competing SA4a, SA4b, SA4c, and SA5 splice sites are multicistronic, potentially coding several proteins from each mRNA (15, 48, 49), we examined the effect of altered proportions of HIV-1 mRNAs on protein expression (Fig. 9). Mutant pNL $\Delta$ SA4b directed the synthesis of a significantly increased level of envelope gp160/120 and Nef but decreased amounts of Rev compared with wild-type provirus (Fig. 9A to C, lanes 3). Because Rev protein plays a prominent role in regulating the level of gp160/120, we evaluated Rev functional activity by measuring the Rev-dependent rescue of CAT activity following cotransfection of pNL $\Delta$ SA4b with the pDM128 Rev reporter plasmid (Fig. 9E and F) (24). Again, Rev activity directed by pNL $\Delta$ SA4b, as measured in this assay, was lower than that directed by wild-type pNL4-3. Thus, the observed elevation of gp160/120 was not due to any increase in Rev activity. Significantly, the expression of both Rev-dependent gp160/120 and Rev-independent Nef proteins were increased in similar proportions. These proteins share SA5 as the predominant competing SA for their mRNAs. Thus, the increased synthesis of gp160/120 and Nef from the pNL $\Delta$ SA4b mutant most likely reflects the increased use of SA5 (and SA4c) and results in

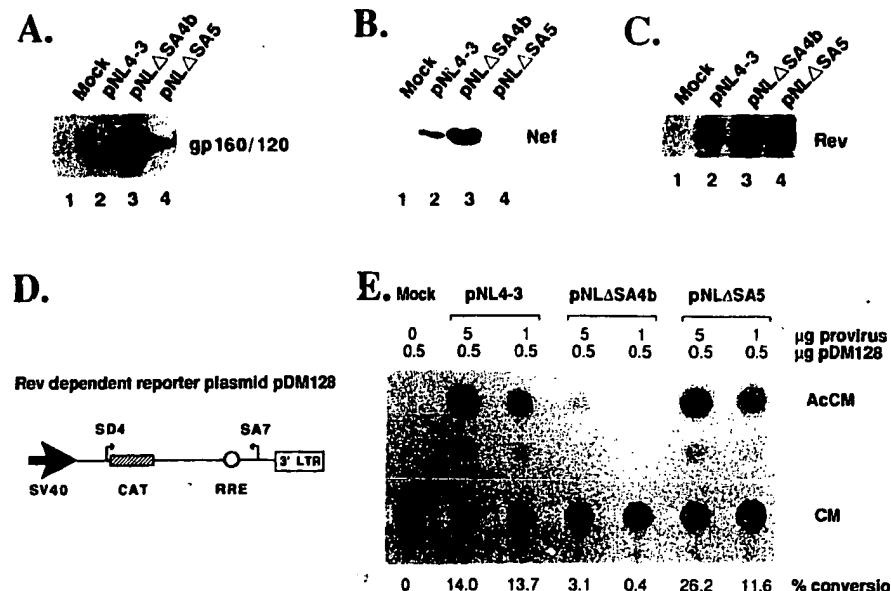


FIG. 9. Translational consequences of alternative usage of competing splice sites by mutant HIV-1 proviruses. Western blot analysis with rabbit serum to gp160/120 (A) or Nef (B) and immunoprecipitation with rabbit serum to Rev from HeLa cell lysates prepared 48 h after cotransfection with 0.5  $\mu$ g of human growth hormone reporter plasmid alone (lane 1) or with 20  $\mu$ g of pNL4-3 (lane 2), pNLΔSA4b (lane 3), or pNLΔSA5 (lane 4) (C). Lysate volumes were standardized according to human growth hormone determination. Functional Rev activity was measured with use of the pDM128 Rev-dependent reporter plasmid of Hope et al. (24) (D) by measuring the amount of CAT activity rescued due to Rev-RRE interaction after cotransfected the indicated amounts of plasmids pDM128 and pNL4-3, pNLΔSA4b, pNLΔSA5 with 0.5  $\mu$ g of human growth hormone reporter plasmid (E). The percentage of chloramphenicol (CM) converted to the acetylated forms (AcCM) is shown at the bottom. SV40, simian virus 40 promoter and enhancer; LTR, long terminal repeat.

increased synthesis of both proteins. This occurs despite the loss of mRNA species using SA4b which encode Rev. Since increased use of SA4c does not prevent the reduction of Rev expression, it is likely that multicistronic mRNAs using SA4c are less efficient for Rev expression than for Nef or gp160/120 expression.

HeLa cells transfected with pNLΔSA5 express very low levels of gp160/120 and Nef proteins but elevated levels of Rev compared with wild-type pNL4-3 (Fig. 9A to C, lanes 4). Elevated Rev activity was also measured in the assay of Rev-dependent rescue of CAT activity, showing that the low level of gp160/120 expression did not result from any deficiency in Rev function. The low-level expression of gp160/120 most likely results from the inefficient translation of the multicistronic env2, env3, and env4 mRNAs, which are present at relatively increased levels in cells transfected with pNLΔSA5 compared with the wild-type pNL4-3 (Fig. 8B, lanes 1 and 3). This result indicates that the multicistronic env mRNAs are markedly less competent for gp160/120 expression than is env1. pNLΔSA5 also fails to synthesize the major nef RNAs (nef2, through nef5; Fig. 4), although the levels of the nef1 cDNA species, which results from splicing of SD1 directly to constitutive SA7, were equivalent in the pNLΔSA5- and wild-type-transfected cells (Fig. 8A). This low expression of Nef protein indicates that the increased amount of bicistronic rev/nef and env/nef mRNAs encoded by mutant pNLΔSA5 fails to compensate for the loss of the predominant monocistronic nef mRNAs. The elevated levels of Rev protein observed demonstrate that the bicistronic rev/nef and env/nef mRNAs encode Rev with significantly higher efficiency than Nef.

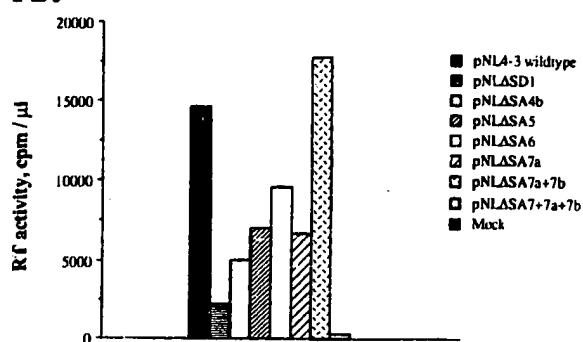
**Infectivity of splicing mutants of HIV-1.** The ability of the HIV-1 splice site mutants to generate progeny virions was assessed by measuring the RT activity released into the medium following cotransfection of HeLa cells with proviral

and human growth hormone DNAs (Fig. 10A). All of the splice site mutants except pNLΔSA7a+7b generated less particle-associated RT than did the wild-type plasmid pNL4-3; pNLΔSA7+7a+7b and pNLΔSD1 produced only 2 and 15% of viral progeny, respectively, compared with the wild type.

The infectivity of the splice site mutants was evaluated by inoculating CEM (12D7) cells with equal amounts of virus harvested from transfected HeLa cell supernatants as determined by RT assay (for wild-type pNL4-3, the multiplicity of infection was approximately 0.002) (Fig. 10B). Spreading infection was established by one of the two proviruses containing a mutated constitutive site. Mutant pNLΔSD1 was infectious but exhibited delayed replication kinetics compared with wild-type pNL4-3, reflecting the delayed kinetics of protein synthesis by this mutant (Fig. 10B). Lower efficiency of RNA splicing from the cryptic donor activated in pNLΔSD1 is very likely responsible for the delayed replication and infectivity kinetics. Mutant pNLΔSA7+7a+7b, which lacks the constitutive SA7 site, could not infect CEM (12D7) cells, reflecting its inability to produce Rev and the HIV-1 structural proteins.

Mutations affecting competing SA sites in HIV-1 had different effects on viral infectivity. Mutant pNLΔSA4b, which fails to generate several HIV-1 mRNAs (rev1, rev4, rev7, and rev10 and env2, env6, env10, and env14) and consistently expressed elevated levels of gp160/120, exhibited growth kinetics similar to the wild-type virus kinetics (Fig. 10B). In this case, altered splicing of viral mRNA had no effect on viral infectivity. Mutant pNLΔSA5, which lacks the processing site for the major env1 mRNA species, was not infectious despite being able to synthesize low levels of gp160/120 in transfected HeLa cells by utilizing alternative SAs (Fig. 6A, lane 4; Fig. 9A). These contrasting results demonstrate that alternative splicing to competing SA sites can affect gp160/120 production, leading to synthesis of either fully infectious or defective virion.

A.



B.

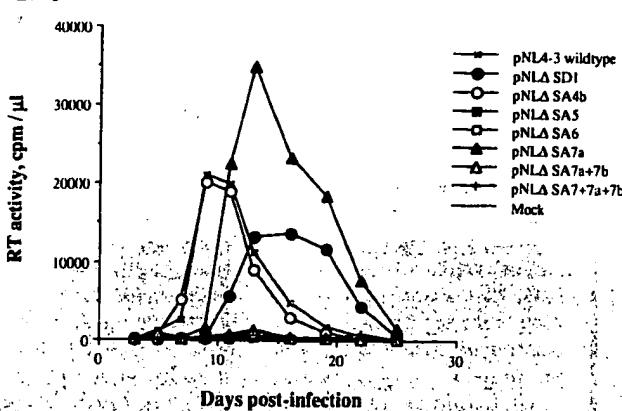


FIG. 10. Efficiency of virion production and infectivity of HIV-1 splicing mutants. (A) Particle production by HIV-1 RNA splicing mutants was measured by using the accumulation of RT activity 40 h after cotransfected HeLa cells with 20  $\mu$ g of mutant provirus plasmids and 0.5  $\mu$ g of human growth hormone reporter plasmid. The RT activity was standardized for transfection efficiency by using the ratio of human growth hormone compared with wild-type pNL4-3 virus. (B) Kinetics of infection of HIV-1 RNA splicing mutants following transfer of cell-free virion obtained from transfection supernatants ( $10^5$  cpm from the RT assay) to CEM (12D7) cells.

The infectivity of mutants affecting the cryptic splice sites was more subtle and could not be predicted from their ability to generate virus particles in the transfection experiments shown in Fig. 10A. Mutant pNLΔSA7a was infectious, but peak virus production was delayed 4 to 5 days compared with the wild type. Mutant pNLΔSA7a+7b, which directed large amounts of progeny virion production following transfection of HeLa cells, was not infectious. Mutant pNLΔSA6 was also not infectious, despite directing the synthesis of the wild-type complement and quantities of protein and spliced mRNAs (Fig. 6 and 7). These latter results could indicate that the cryptic SA6 and SA7b sites participate in some other aspect of RNA processing (e.g., folding or branch point formation). Alternatively, amino acid changes introduced into the envelope protein as a result of mutagenesis (see Materials and Methods) may eliminate virus infectivity, possibly by affecting gp160 processing as suggested by reduced amounts of gp120 observed in Fig. 6A. In a previous study, a similar mutation of SA6 in the HIV-1<sub>11XB2</sub> isolate resulted in the loss of infectivity, whereas SD5 mutants, also defective for Rev expression, had a wild-type phenotype (16).

## DISCUSSION

**Relative proportions of alternatively spliced HIV-1 mRNAs.** Various regulatory mechanisms control the expression and function of HIV-1 during a cycle of virus infection. The regulation of RNA processing is one such prominent mechanism, and the balanced splicing of genomic length RNA into a complex set of alternative RNA transcripts is required for the synthesis of several viral proteins essential for replication. Several transcripts are capable of expressing each of the regulatory and accessory HIV-1 proteins, and most of these transcripts have the potential to encode two or more proteins with different efficiencies. To evaluate the importance of the complex group of mRNAs synthesized during infection by HIV-1, we first rigorously determined the identities and relative quantities of viral mRNAs resulting from transfection and infection experiments. Our analysis shows that some RNA species are synthesized in preference to others. Generally, the most highly spliced forms of RNA that exclude noncoding exons are most common except in the case of *nef*, in which case the inclusion of the 68-nt noncoding exon 5 is favored. A previously unrecognized SA site for *rev*- and *env/vpu*-encoding mRNAs (designated SA4c) was identified among a cluster of competing SA sites in the *tat* coding sequence. This site was selected at fivefold-lower frequency than the SA4a or SA4b site for both *rev*- and *env*-mRNAs in PBMC infected with HIV-1<sub>NL4-3</sub> or HIV-1<sub>AI</sub>. The SA4c site is used by many strains of HIV-1 and is the predominant SA used for *rev*-mRNA by some HIV-1 strains (42). The addition of the new SA4c site to the central competing SA sites (SA4, SA4a; SA4b; SA4c, and SA5) determined that 16 alternative mRNAs may encode gp160/120. However, most of these exist at very low levels, and the most common *env*-mRNAs either used SA5 or excluded both noncoding exons 2 and 3. The shortest possible *env* transcript (*envl*) accounts for 80% of all *env* RNA directed by HIV-1<sub>NL4-3</sub>.

**Competing SA site usage determines gp160/120 levels and virus infectivity.** Changing the balanced usage of competing splice sites caused alterations in the proportions of both RNA and protein species and, in some cases, viral infectivity. HIV-1 mutants containing changes affecting SA4b gave rise to an increased proportion of the mRNA species using neighboring SA4a; SA4c, and SA5. This caused elevated expression of envelope gp160/120 but no loss of virus infectivity. Increased synthesis of HIV-1 *env*-mRNA using SA5, which more efficiently yields gp160/120, is likely to explain the increase in gp160/120. Mutation of the closely adjacent SA5, the major SA for *env*-mRNA, resulted in increased use of SA4a, SA4b, and SA4c but was accompanied by a marked reduction in both the expression of envelope gp160/120 and virus infectivity. The reduced levels of gp160/120 were not associated with reduced Rev activity, and Gag protein production was not altered. These results demonstrate that the level of expression of envelope proteins can be dramatically altered by forcing different splicing patterns on HIV-1<sub>NL4-3</sub> through the disproportionate usage of the seemingly redundant SA sites in this region of the viral genome.

Two determinants could control the selection of the competing SA sites if this alternative splicing mechanism were to operate in vivo. First, the different sequence structure of the competing SA motifs (Fig. 3) or the branch point structure(s) in individual HIV-1 strains could alter the balance of the SA usage. The location of the branch point(s) for the competing HIV-1 SA sites is unknown. We have confirmed that HIV-1 strains with different sequences have different splicing patterns in a survey of various HIV-1 isolates exhibiting variable

tropism (42). Second, the activation status or type of cell harboring an HIV-1 provirus could also affect the balance of SA usage.

Our analysis of splice site mutants suggest that an alteration in the balanced selection of the competing SA sites may have a profound effect on the ability of some HIV-1-infected cells to produce infectious virion. The potential deficit of gp160/120 protein resulting from underutilization of SA5 in a wild-type HIV-1 isolate may reduce the infectivity of viral progeny by interfering with adsorption-penetration step of the virus life cycle. This mechanism may contribute to some of the noninfectious virus present in the plasma of infected individuals (4, 11, 39, 41).

**Biological significance of multicistronic HIV-1 mRNAs.** Many of the alternative HIV-1 mRNAs are multicistronic, encoding more than one protein when tested in *in vitro* translation systems (15, 47). Some of the splice site mutants described above selectively eliminate several of these mRNAs from the HIV-1 RNA pool, allowing an evaluation of these depleted transcripts in the context of virus replication. The inactivation of SA5 from provirus mutant pNL $\Delta$ SA5 precluded the synthesis of the major species of *env* mRNA (*env1*) as well as the predominant *nef* mRNAs (*nef2* through *nef5*). During productive infection of PBMC with wild-type HIV-1, *env1* and *nef2* through *nef5* mRNAs comprise 80% of the total *env* and 95% of the total *nef* transcripts, respectively. Intuitively, the elimination of these mRNAs could be compensated for by the increased use of other neighboring SAs which could be used to synthesize alternate multicistronic mRNAs with coding potential for the deficient Env and Nef proteins. Our analyses showed that multicistronic mRNAs generated only limited amounts of Nef and gp160/120 proteins which proved inadequate for the production of infectious progeny virion. After transfection of HeLa cells with mutant pNL $\Delta$ SA5, minimal synthesis of gp160/120 (~2% of the wild-type level) and low levels of Nef (~12% of the wild-type level) were measured. In our studies, bicistronic *rev* mRNAs encoded Nef protein with 2- to 3-fold-lower efficiency than did monocistronic *nef* mRNAs (in agreement with results of Schwartz et al. [49]) and gp160/120 was translated from *env1* mRNA approximately 25-fold more efficiently than from other *env* mRNAs. Given the proportions of the different HIV-1 mRNAs measured in this study, neither multicistronic *env* RNA containing the Rev, Vpu, and Env translation initiation sites (*env2* through *env5*) nor the multicistronic *rev* RNA species, also capable of directing the synthesis of Nef (15, 47), significantly contributes to the total cellular pool of gp160/120 or Nef proteins during virus replication. In contrast, a mutation affecting one of the SAs for *rev* mRNA (*SA4b*) resulted in *rev* mRNAs using *SA4a* and *SA4c* that fully compensated for altered SA usage and produced wild-type levels of Rev functional activity.

**Mutation of constitutive splice sites activates cryptic sites.** Mutation of the major SD (SD1) in the pNL4-3 provirus slowed the kinetics of RNA and protein synthesis and the kinetics of a spreading virus infection. An alternative cryptic major SD signal, four bases downstream, was activated. Because the cryptic SD1 site may be used only when the genuine site is inactivated, it is likely that the strong conservation of this alternative splice site results from additional selective pressures. Nevertheless, its existence greatly reduces the possible loss of virus infectivity due to a spontaneous mutation affecting the major SD that would otherwise block the production of functional spliced mRNAs. The efficiency of RNA splicing from this cryptic donor ( $_{-3}$ AGA  $\downarrow$  GUACGCC $_{+7}$ ) may be lower than that from the genuine donor site ( $_{-3}$ CUG  $\downarrow$  UGAGUAC $_{+7}$ ) and would therefore be responsible for the

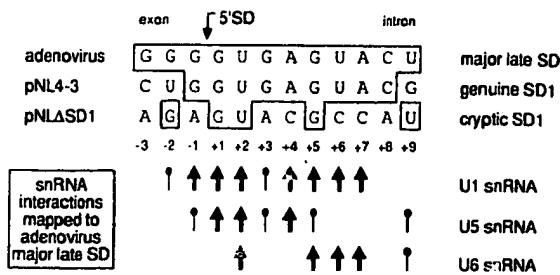


FIG. 11. HIV-1 SD1 has greater potential to interact with U1, U5, and U6 snRNAs than does the cryptic SD1. Shown is alignment of the SD site of adenovirus major late pre-mRNA with the genuine and cryptic SD1 sites of pNL4-3 and pNL $\Delta$ SD1; homologous nucleotides are boxed. Locations of the adenovirus nucleotides identified by Wasserman and Steitz (56) as strongly (↑) or weakly interacting (↑) with U1, U5, and U6 snRNAs are shown at the bottom.

delayed replication and infection kinetics of mutant pNL $\Delta$ SD1. Comparison of these two HIV-1 SD motifs with the adenovirus major late 5' SD ( $_{-3}$ GGG  $\downarrow$  GUGAGUA $_{+7}$ ) (Fig. 11), used to map nucleotide interactions with U1, U5, and U6 small nuclear RNAs (snRNAs), provides an explanation for the possible lower splicing efficiency of the cryptic SD1 (56). The nucleotides at sites of strong interaction between the adenovirus SD and the U1, U5, and U6 snRNAs of the spliceosome are the same for wild-type HIV-1 SD1 but mismatched at several positions for the cryptic HIV-1 SD1, potentially destabilizing the interaction of the cryptic SD1 with the spliceosome (Fig. 11). The decreased affinity of the cryptic HIV-1 SD with several important spliceosomal snRNAs may explain the delayed infection kinetics of mutant pNL $\Delta$ SD1.

Mutation of SA7, in conjunction with SA7a and SA7b, resulted in the expression of aberrant RNA and protein species and the loss of virus infectivity as a consequence of the activation of a downstream cryptic SA site. The use of this latter site shifted the reading frame for the second coding exon of Rev, thereby causing a concomitant loss of Rev function. Despite the apparent lack of expression of functional Rev by mutant pNL $\Delta$ SA7a+7b, all other HIV-1 SD and SA site usage was equivalent to that of wild-type pNL4-3, indicating that the two-exon Rev protein is not required for the selection of splice sites in HIV-1 RNAs with or without an RRE. However, assembly of the spliceosome may still be required for Rev to activate RNA containing an RRE (6).

**Significance of cryptic splice sites in HIV-1 replication.** We found that three previously mapped splice acceptor sites, SA6, SA7a, and SA7b (47), were never used during HIV-1<sub>pNL4-3</sub> replication in PBMC and HeLa cells and that mutation of these cryptic sites had no apparent effect on HIV-1 RNA or protein synthesis. These sites were unlikely candidates as commonly used ligation points for the synthesis of mature mRNA because HIV-1 isolates, other than derivatives of HIV-1<sub>LA1</sub>, do not possess the SA6 splicing signal (37), and the use of SA7a and SA7b sites would preclude the in-frame entry into the second coding exon of *rev* and *tat*, leading to the synthesis of novel Rev-Tat-Env chimeric translation products and no functional Rev protein. The ability of mutant pNL $\Delta$ SA7a to successfully replicate and infect human cells further demonstrates that this site is not essential in the HIV-1 life cycle. Unexpectedly, mutants pNL $\Delta$ SA6 and pNL $\Delta$ SA7a+7b were not infectious, suggesting that these sites might play some other role in RNA processing or that the amino acid structure around these mutagenized SAs is critical for infectivity. Mutational analysis of the *rev* splicing signals (SD5) in

the context of HIV-1<sub>IIIB</sub> indicated that Tev was not required for virus infections of CD4<sup>+</sup> T-cell lines and activated PBMC. However, the *tev* splicing signal (SA6) in HIV-1<sub>IIIB</sub> stabilized neighboring suboptimal splice sites and maintained the balanced proportion of spliced and unspliced RNAs (16). The *tev* splicing pattern may be limited to certain derivatives of HIV-1<sub>AI</sub> and could have arisen as a result of inefficient splicing signals throughout the primary RNA transcript in this isolate.

In contrast to the uncertain role of cryptic splice sites in HIV-1, changing the usage of the competing splice sites in the center of the HIV-1 genome caused alterations in the proportions of both HIV-1 RNA and protein species, and this affected viral infectivity. Similarly, mutation of the major SD motif markedly reduced the synthesis of viral RNA and protein as well as viral infectivity. Therefore, we have shown that alternative splicing of mature HIV-1 mRNA is yet another potential mechanism for the regulation of HIV-1 expression. Thus, the multiple redundant splice signals in the central region of the HIV-1 genome play a greater role than simply providing redundant strategies for mRNA synthesis in the event of unwanted mutations at these sites.

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## Characterization and Expression of Novel Singly Spliced RNA Species of Human Immunodeficiency Virus Type 1

SALVATORE J. ARRIGO,<sup>1</sup> STACY WEITSMAN,<sup>1</sup> JEROME A. ZACK,<sup>1</sup> AND IRVIN S. Y. CHEN<sup>1,2\*</sup>

Department of Microbiology and Immunology,<sup>1</sup> and the Department of Medicine,<sup>2</sup> UCLA School of Medicine and Jonsson Comprehensive Cancer Center, Los Angeles, California 90024-1678

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Human immunodeficiency virus type 1 (HIV-1) expresses the Vif, Vpr, Vpu, and Env proteins through complex differential splicing of a single full-length RNA precursor. We used HIV-1-specific oligonucleotide primer pairs in a quantitative polymerase chain reaction procedure on RNA from fresh peripheral blood lymphocytes infected with HIV-1<sub>JR-CSF</sub> to detect and characterize the singly spliced RNA species which might encode these proteins. The nucleotide sequences at the junctions of splice donor and acceptor sites of these RNAs were determined. One of these RNAs, which has not been previously described, appears to be a novel HIV-1 RNA encoding Env and/or Vpu proteins.

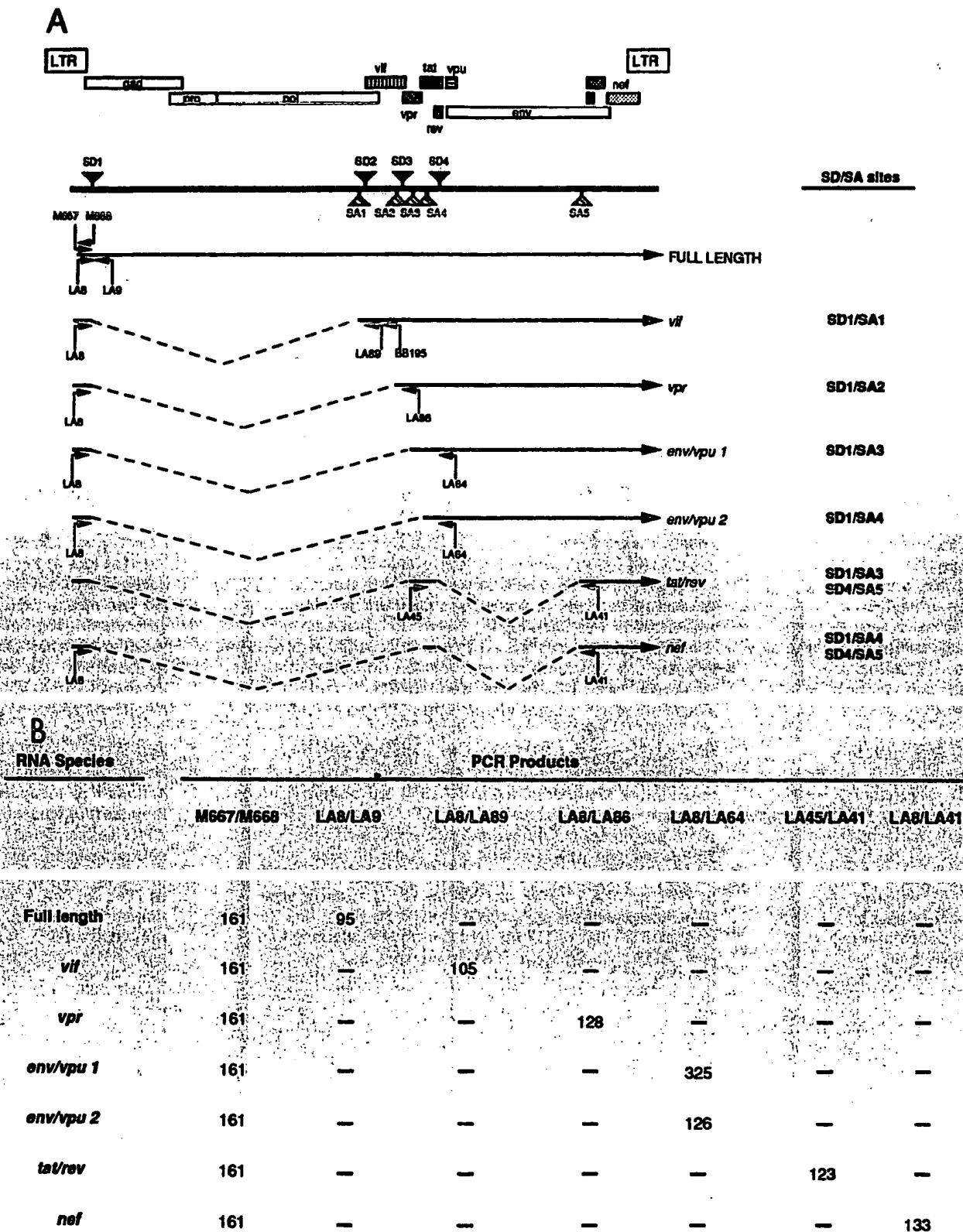
In addition to *gag*, *pol*, and *env*, the three genes found in all replication-competent retroviruses, human immunodeficiency virus type 1 (HIV-1) has at least seven additional genes, including *tat*, *rev*, *nef*, *vif*, *vpr*, *env*, and *vpu* (3, 5, 9, 11-16). Although the mRNAs with the potential to express some of these genes have been elucidated (1, 3, 7, 8, 10, 14), the RNAs encoding Vif, Vpr, Env, and Vpu proteins have not been clearly defined. Several 4.2- to 5.5-kilobase-spliced RNA species that might encode these proteins have been detected by Northern (RNA) blot analysis (8). One of these RNAs with the potential to encode Env and Vpu proteins has been characterized by sequence analysis of cDNA clones (7). Another partial cDNA clone with the potential to encode Vif has also been characterized (14). Potential splice donors (SDs) and splice acceptors (SAs) which might be used in the splicing of these RNAs have been identified only in small doubly and triply spliced HIV-1 RNAs by cDNA cloning (1, 3, 7).

**Oligonucleotides for the detection of HIV-1 RNAs.** We generated HIV-1-specific oligonucleotide pairs to use in a quantitative RNA polymerase chain reaction (PCR) procedure for detection of singly spliced RNAs which might encode Vif, Vpr, Env, and Vpu proteins (Fig. 1A). We previously used this RNA PCR procedure to quantitatively detect HIV-1 *tat/rev*, *nef*, and full-length *gag/pol* RNAs, as well as total HIV-1 RNA (2). The oligonucleotide primer pairs to detect singly spliced RNAs were designed to flank potential SD-SA junctions. Vif, Vpr, Env, and Vpu proteins should be translated from RNAs which have utilized the 5' SD (SD1) at nucleotide (nt) 743 of HIV-1<sub>JR-CSF</sub>. On the basis of sequencing of cDNA clones, potential SAs for *vif* and *vpr* RNAs have been postulated to reside at nt 4924 (SA1) and nt 5401 (SA2) (7, 14). However, the original cDNA clones in which these SAs were detected were derived from RNAs which excised three introns, additionally utilizing the SDs at nt 4973 (SD2) and 5474 (SD3), and thus could not encode Vif or Vpr proteins. A partial cDNA clone which might encode Vif, utilizes SA1, and extends unspliced past SD2 and SD3 has been described previously (14). One cDNA clone derived from an RNA which might encode Vpu and Env has been described previously (7). This RNA uses an SA at nt

5788 (SA3). We generated 3' oligonucleotide primers LA89 (nt 4995 to 4976), LA86 (nt 5495 to 5476), and LA64 (nt 6079 to 6060) to detect RNAs which utilize the known SAs of HIV-1 and which might encode the Vif, Vpr, Env, and Vpu proteins of HIV-1 (Fig. 1). These oligonucleotide primers, when used in PCR analysis with LA8 (nt 711 to 730) as the 5' oligonucleotide primer, should detect HIV-1 RNAs which splice together the leader sequence of HIV-1 with any SA located upstream of the 3' oligonucleotide primer. Any RNAs that utilize additional SDs and SAs which flank the 3' oligonucleotide primer will not be detected. The sizes of PCR products that should be generated by these oligonucleotide primers on the various singly spliced RNAs are shown in Fig. 1B.

**Quantitative detection of novel HIV-1 RNAs.** RNA was prepared from phytohemagglutinin-stimulated peripheral blood lymphocytes (PBL) 8 days subsequent to infection with HIV-1<sub>JR-CSF</sub> (4). This RNA was subjected to treatment with RNase-free DNase to remove all contaminating DNA and was used in RNA PCR analysis with oligonucleotide primer pairs to detect *vif*, *vpr*, and *env/vpu* RNAs. Oligonucleotide primers LA89 (nt 4995 to 4976), LA86 (nt 5495 to 5476), and LA64 (nt 6079 to 6060) were used in combination with oligonucleotide primer LA8 (nt 711 to 730) for detection of *vif*, *vpr*, and *env/vpu* RNAs, respectively. RNA was sequentially diluted threefold from  $10^5$  to  $4 \times 10^2$  cell equivalents prior to reverse transcription and PCR amplification. Figure 2 shows that HIV-1-specific *vif*, *vpr*, and *env/vpu*-2 RNAs can be detected quantitatively in HIV-1-infected PBL. The sizes of major bands generated by PCR amplification with these oligonucleotide primer pairs correspond directly to the predicted sizes of the products expected from the joining of the SDs and SAs shown in Fig. 1. These bands were not detected in uninfected PBL (data not shown). By using oligonucleotide pairs LA8 (nt 711 to 730) and LA64 (nt 6079 to 6060) to detect RNAs which could encode Env or Vpu, we detected a novel spliced RNA (*env/vpu*-2), which utilizes the joining of SD1 (nt 743) with an SA at nt 5987 (SA4). This RNA does not utilize SD4 (nt 6055), which is used in further splicing to *nef* RNA. The first and second methionines encountered within this novel RNA would be the initiation codons for Vpu and Env, respectively. This is in contrast to the previously identified *env/vpu*-1 RNA, in which *vpu* and *env* utilize the third and fourth

\* Corresponding author.



**FIG. 1. Detection of HIV-1 RNAs by PCR.** (A) Schematic representation of the HIV-1 genome showing the putative RNAs and the location and orientation of oligonucleotide primer pairs used to detect each RNA. The locations of potential SDs and SAs are indicated. Oligonucleotide primer LA8 (5'-GCGCGCACAGCAAGAGGCGA-3', nt 711 to 730) is homologous to HIV-1 sequences just upstream of the 5' SD site (SD1 at nt 743). It can be used in PCR analysis in combination with oligonucleotide primer LA89 (5'-TGTATTACTACTGC CCCITC-3', nt 4995 to 4976) on the cDNA products of reverse transcription reactions primed by oligonucleotide primer BB195 (5'-TATGTCACTATTATCTTGTAA-3', nt 5011 to 4992) to detect a potential *vif* RNA which utilizes the joining of SD1 (nt 743) with SA1 (nt

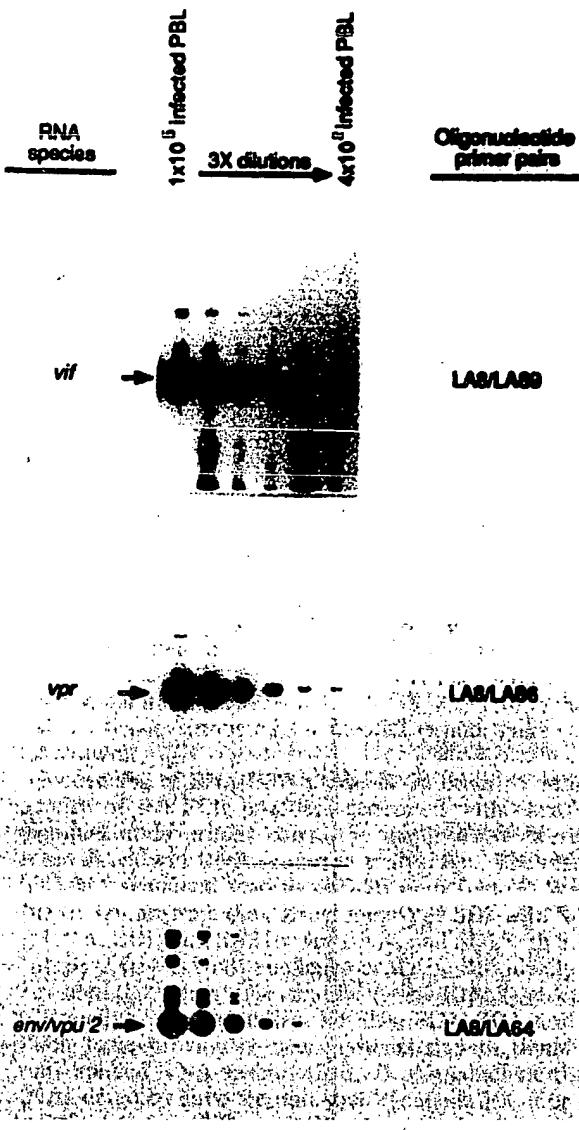
methionines as initiation codons. Minor bands were observed with several of the oligonucleotide pairs tested. DNA sequence analysis of several of these bands has shown that some of these bands are due to nonspecific hybridization to both cellular sequences and other sites within HIV during reverse transcription (data not shown). Some of these bands may represent minor species of RNA produced during infection of PBL.

We have not successfully detected the singly spliced RNA joining SD1 to SA3 (*env/vpu-1*) previously observed by cDNA cloning. Since the *tat/rev* and *env/vpu-1* RNAs both employ SA3, the 3' oligonucleotide primer that discriminates *env/vpu-1* from *tat/rev* RNAs must be positioned 3' to SD4 (used only by *tat/rev* RNAs); thus, oligonucleotide primer LA64 (nt 6079 to 6060) was used. Both *env/vpu-1* and *env/vpu-2* RNAs would be predicted to be detected by the LA8-LA64 oligonucleotide primer pair (nt 711 to 730 and nt 6079 to 6060); however, perhaps due to the large size (325 base pairs) of the anticipated PCR product for *env/vpu-1* RNA, only the smaller PCR product for *env/vpu-2* RNA is detected. The inability to detect this larger *env/vpu* RNA does not suggest that this RNA is not produced, since we have previously shown that when oligonucleotide primer pairs are used in this RNA PCR procedure, which should detect more than one RNA species, only the smaller PCR product is readily detected (2). We have also been unable to detect a distinct RNA species encoding Rev (10) in infected PBL by using various oligonucleotide primer pairs (2; data not shown).

**Sequencing of RNA PCR products.** In order to verify the identity of the PCR products generated with each set of oligonucleotide primer pairs, the major bands were subjected directly to DNA sequence analysis (6). The results of this DNA sequence analysis are shown in Table 1. We were able to demonstrate the correct joining of SD1 with SA1 for *vif* RNA, SD1 with SA2 for *vpr* RNA, and SD1 with SA4 for *env/vpu-2* RNA. DNA sequence analysis additionally demonstrated that the *vif*, *vpr*, and *env/vpu-2* RNAs detected by PCR analysis do not utilize the SDs, SD2, SD3, and SD4, which have previously been associated with doubly and triply spliced RNA species (1, 3, 7). We conclude that the RNAs detected have the potential to encode Vif, Vpr, Env, and Vpu proteins. These RNAs most likely correspond to the 4.2- to 5.5-kilobase HIV-1 RNAs detected by Northern blot analyses (8).

We generated oligonucleotide primer pairs to be employed in a quantitative RNA PCR procedure to detect *vif*, *vpr*, and *env/vpu-2* HIV-1-specific RNA species. We identified RNAs which join the 5' HIV-1 SD (SD1) with previously detected SAs (SA1, SA2, and SA4), which have the potential to encode Vif, Vpr, Env, and Vpu proteins. Although usage of these SAs has been previously demonstrated by sequencing of cDNA clones (7), the RNAs which had been detected were, in all cases, additionally spliced at SD2, SD3, and SD4. A partial cDNA clone which utilizes SA1 but not SD2

4924). It will not detect an RNA which additionally utilizes SD2 (nt 4973). The use of different oligonucleotides in the reverse transcription and PCR amplifications helped to reduce the background with this oligonucleotide set. Oligonucleotide primer LA8 can also be used in RNA PCR analysis in combination with oligonucleotide primer LA86 (5'-CAAGTACTGTAGAGATCCTA-3', nt 5495 to 5476) to detect a potential *vpr* RNA that utilizes the joining of SD1 (nt 743) with SA2 (nt 5401), but it will not detect an RNA which additionally utilizes SD3 (nt 5474). Oligonucleotide primer LA8 can also be used in an RNA PCR in combination with oligonucleotide primer LA64 (5'-GGTTGCATTACAT GCACTAC-3', nt 6079 to 6060) to detect potential *env/vpu* RNAs which utilize the joining of SD1 (nt 743) with SA3 (nt 5788) or SA4 (nt 5987) but do not utilize SD4 (nt 6055). SD4 (nt 6055) is used by both *tat/rev* and *nef* RNAs. Oligonucleotide primers M667, M668, LA9, LA45, and LA41 have been previously described (2). The SD and SA sites used to generate each RNA are shown. LTR, long terminal repeat. (B) Sizes of amplified PCR products which should be generated by RNA PCR analysis with pairs of oligonucleotide primers specific for the HIV-1 RNAs shown in panel A.



**FIG. 2.** Quantitative detection of HIV-1 RNAs. RNA PCR analysis was performed on cell equivalents of RNA from the indicated number of PHA-stimulated PBL harvested on day 8 after infection with HIV-1<sub>LR-CSF</sub>. RNA was prepared by the method of Arrigo et al. (2). The same conditions for RNA PCR analysis as those described by Arrigo et al. were also used, with the exception that the PCR denaturation temperature was changed to 94°C. The PCR products were resolved on a 6% polyacrylamide gel and visualized by autoradiography. Oligonucleotide primer pairs used to detect each RNA species are shown. The sections of the gels shown span approximately 70 to 350 base pairs, as determined by molecular weight markers.

TABLE 1. Sequences of SDs and SAs<sup>a</sup>

RNAs (oligonucleotide primer pairs)		Sequence	
<i>vif</i> (LA8/LA89; nt 711 to 730, 4995 to 4976)	SD1 GGGGCGGCGACTG (743)	GTGAG.....TTTATTACAG	SA1 GGACAAACA (4924)
<i>vpr</i> (LA8/LA86; nt 711 to 730, 5495 to 5476)	SD1 GGGGCGGCGACTG (743)	GTGAG.....TGTTTTTCAG	SA2 AATCTGCT (5401)
<i>env/vpu-2</i> (LA8/LA64; nt 711 to 730, 6079 to 6060)	SD1 GGGGCGGCGACTG (743)	GTGAG.....CCTATGGCAG	SA4 CAAGAACG (5987)

<sup>a</sup> PCR amplifications were resolved on a 6% polyacrylamide gel, and the major band was excised. DNA was eluted in 500 mM ammonium acetate and 1 mM EDTA and was precipitated with ethanol. A fraction of this eluent was reamplified for an additional 25 cycles of PCR. PCR conditions for both amplifications were identical, although the second amplification included a fourfold increase in all reagents. Since the PCR products were end labeled with [ $\gamma$ -<sup>32</sup>P]ATP, a portion of the PCR product was subjected directly to DNA sequence analysis by the method of Maxam and Gilbert (6). The sequences include SDs, introns, and SAs, from left to right. The vertical line indicates the exon-intron borders. Numbers in parenthesis shown under nucleotide sequences correspond to the nucleotides to the immediate left (SD) or right (SA) of the vertical lines.

or SD3 has been previously characterized (14). It is a formal possibility that the RNAs we detected for *vif*, *vpr*, and *env/vpu-2* contain additional splices downstream of the regions homologous to our oligonucleotides; however, we feel this is unlikely, because of the 4.2- to 5.5-kilobase sizes of these HIV-1 RNA species previously detected by Northern blot analyses with probes specific for these RNAs (8). Taken together, these data indicate that *Vif*, *Vpr*, *Env*, and *Vpu* are most likely encoded on singly spliced RNA species 4.2 to 5.5 kilobases in size by utilizing the SD and SA sites which we define here.

This RNA PCR procedure provides an extremely sensitive and quantitative assay for analysis of low abundance RNA species, allowing us to analyze expression of HIV-1 RNAs at early time points subsequent to infection of PBL. By generating oligonucleotide primers which span potential SD and SA pairs, specific RNA species can be examined individually and the DNA sequence of the splice junctions can be determined directly.

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## Requirements for Efficient Production and Transduction of Human Immunodeficiency Virus Type 1-Based Vectors

MEHDI GASMI, JACQUELINE GLYNN, MING-JIE JIN, DOUGLAS J. JOLLY, JIING-KUAN YEE, AND SHIN-TAI CHEN\*

*Center for Gene Therapy, Chiron Technologies, San Diego, California 92121*

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A number of human immunodeficiency type 1 (HIV-1)-based vectors have recently been shown to transduce nondividing cells *in vivo* as well as *in vitro*. However, if these vectors are to be considered for eventual clinical use, a major consideration is to reduce the probability of unintended generation of replication-competent virus. This can be achieved by eliminating viral genetic elements involved in the generation of replication-competent virus without impairing vector production. We have designed a system to transiently produce HIV-1-based vectors by using expression plasmids encoding Gag, Pol, and Tat of HIV-1 under the control of the cytomegalovirus immediate-early promoter. Our data show that the best vector yield is achieved in the presence of the Rev/Rev-responsive element (RRE) system. However, the constitutive transport element of Mason-Pfizer monkey virus can substitute for RRE and Rev at least to some extent, whereas the posttranscriptional regulatory element of human hepatitis B virus appeared to be inefficient. In addition, we show that high-titer virus preparations can be obtained in the presence of sodium butyrate, which activates the expression of both the packaging construct and the vector genome. Finally, our results suggest that efficient infectivity of vectors defective in the accessory proteins Vif, Vpr, Vpu, and Nef depends on the nature of the target cells.

Vectors derived from Moloney murine leukemia virus (MLV) are widely used in gene delivery and human gene therapy studies. Most mammalian cells express the MLV amphotropic receptor on the cell surface, allowing vector entry (8). However, the nuclear entry of the vector preintegration complex depends on cell mitosis, probably due to nuclear membrane breakdown (25). Thus, MLV-based vectors efficiently infect only proliferating cells and not quiescent cells. This property severely limits the general use of retroviral vectors for direct gene delivery *in vivo*, since a majority of the cells either are terminally differentiated or remain in the quiescent state without stimulation. In contrast to MLV, lentiviruses can infect and integrate their genomes into the chromosomes of nondividing cells. In the case of human immunodeficiency virus type 1 (HIV-1), the capability of infecting quiescent cells maps to three viral proteins present in the HIV-1 preintegration complex, namely, the matrix (MA) portion of the Gag protein, the integrase, and the Vpr protein (7, 11-13, 18). To date, the relative importance of these apparently redundant functions is unknown. Recently, HIV-1-based vectors pseudotyped with heterologous envelopes, notably the G protein of vesicular stomatitis virus (VSV), have been shown to infect a wide array of quiescent cell types, including fibroblasts and primary monocyte-derived macrophages in culture as well as hepatocytes, myocytes, photoreceptor cells in retina, and neuronal cells in brain *in vivo* (5, 22, 29, 30, 46). MLV vectors, under similar conditions, fail to infect these cell types efficiently. These observations demonstrate the potential advantages of using lentivirus vectors for direct *in vivo* gene delivery.

Most of the HIV-1-based vector production systems reported to date consist of the cotransfection into 293T cells of three plasmid constructs: (i) a packaging construct containing all HIV genes except the *env* gene, (ii) an expression plasmid

for the VSV G protein to confer a broad host tropism to the vector, and (iii) an HIV vector containing the gene of interest. When considering using HIV-1-based vectors for gene therapy, one needs to address the possibility of generating replication-competent HIV-1 during vector production. The major route for generating replication-competent retrovirus with such a system would be through homologous recombination events occurring among these plasmid constructs during transfection. Safety would be greatly improved by deleting regions of the viral genome that are not absolutely required for vector production or for efficient infection of the target cells. In addition, elimination of genes nonessential for HIV vector production and infectivity will facilitate the establishment of stable packaging cell lines for HIV vectors.

Apart from the *env* gene, which is inactivated since the particles are pseudotyped with the VSV G protein, the obvious target sequences to be eliminated are the HIV-1 genes encoding the accessory proteins. The HIV-1 genome encodes two regulatory proteins, Tat and Rev, as well as four accessory proteins, namely, Vif, Vpr, Vpu, and Nef. Although transcription initiation from the HIV long terminal repeat (LTR) depends on Tat, insertion of an enhancer from the cytomegalovirus (CMV) immediate-early gene bypasses the Tat requirement for HIV gene expression (23). However, recent data demonstrates that Tat is essential for the HIV life cycle at a postentry step in the target cell, suggesting that Tat expression in the packaging cell lines not only can increase vector titers but also may enhance infection of the target cells (17, 20). Rev binds the Rev-responsive element (RRE) within the *env* gene in the viral mRNAs and thereby increases transportation of unspliced or singly spliced HIV-specific mRNA from the nucleus into the cytoplasm (9, 26). Although this is an essential function for HIV-1 replication, other RNA transport elements have been shown to substitute for the RRE function (6, 19). Most importantly, the function of these elements relies on endogenous factors rather than on Rev. Bypassing the RRE and Rev requirement may thus eliminate the need for the stable expression of HIV Rev in the packaging cell lines. The Vif, Vpr, Vpu,

\* Corresponding author. Present address: Jerry L. Pettis VAMC and Loma Linda University, Mineral Metabolism (151), 11201 Benton St., Loma Linda, CA 92357. Phone: (909) 422-3101. Fax: (909) 796-1680. E-mail: STChen@netscape.net.

and Nef proteins have been shown to be dispensable for HIV replication in immortalized cell lines (28). In addition, it has recently been shown that none of the accessory proteins is required for efficient HIV-based vector production from transfected 293T cells (22, 23, 46).

In the present study, we evaluated more precisely the requirement for Rev and the accessory proteins, Vif, Vpr, Vpu, and Nef, for the production of high-titer HIV-1-based vectors and efficient transduction of target cells. We have also tested heterologous mRNA transport elements derived from Mason-Pfizer monkey virus (MPMV) and hepatitis B virus (HBV) for their ability to substitute for the function of Rev and RRE. Our results show that while the absence of the accessory proteins has little effect on vector production, the presence of sodium butyrate, which activates the CMV enhancer and the HIV LTR promoter function (24, 39), can significantly increase vector titers from the transfected 293T cells. While the MPMV constitutive transport element (CTE) can substitute for the function of RRE and Rev, the vector can be generated most efficiently only from a packaging plasmid containing the RRE sequence. Finally, while the absence of accessory proteins in the vector shows little effect on infectivity in most cell types, these proteins enhance significantly the infectivity of HIV vector in quiescent primary human skin fibroblasts. Our studies should facilitate the establishment of stable packaging cell lines for the production of high-titer, helper-free HIV vectors for human gene therapy.

#### MATERIALS AND METHODS

**Plasmid construction.** To construct pCMV-HIV-1, the 0.7-kb *Bam*HI-*Sph*I fragment with a 19-bp deletion in the putative packaging signal of pCMVΔP1ΔenvpA (31) was fused with the 8-kb *Sph*I-*Hind*III (from position 1447 to 9606) fragment of pNL4-3 (1) and the 4-kb *Sal*I-*Eco*RI fragment from pCMV-G (45). In addition, a deletion of the 580-bp *Bgl*II (position 7031 in pNL4-3)-*Bgl*II (position 7611 in pNL4-3) fragment was created in the HIV-1 Env-coding region to eliminate the expression of this protein and reduce the potential of generating helper virus during vector production. To generate pCMV-HIV<sub>nef</sub>(-), the sequence between the *Hpa*I site at position 8650 in pNL4-3 and the *Hind*III site at position 9606 in pNL4-3 of pCMV-HIV-1 was deleted. To generate pCMV-HIV<sub>vif</sub>(-), pCMV-HIV-1 was digested with *Nde*I (position 5123 in pNL4-3) and repaired with the Klenow fragment to create a 2-bp insertion in the coding region of the *vif* gene. To generate pCMV-HIV<sub>vpu</sub>(-), the initiation codon of Vpu was mutated by site-directed mutagenesis (Mutagene kit; Bio-Rad, Hercules, Calif.) with the oligonucleotide 5'-TGCTAC TATTATAGTTGATCATGTTACTACTTG3'. To generate pCMV-HIV<sub>vpr</sub>(-), pCMV-HIV-1 was digested with *Eco*RI (position 5747 in pNL4-3) and repaired with the Klenow fragment to generate a 4-bp insertion in the coding region of the *vpr* gene. The pCMV-HIV<sub>vpr</sub>(-)<sub>nef</sub>(-) double mutant was generated by digesting pCMV-HIV<sub>nef</sub>(-) with *Eco*RI and repaired with the Klenow fragment as described above.

To generate pCH-GP-1, the 0.66-kb fragment between position 766 and the *Sph*I site at position 1447 in pNL4-3 was amplified by PCR with the oligonucleotides Gag5' (5'GAGGATCTAGAAGGAGAGATGGT3') and Gag3' (5'GAGGATCCAATAGGCCCTGCATGCACTG3'). The resulting fragment was ligated with the 3.7-kb *Sph*I-*Nde*I fragment from pNL4-3 and the 4-kb *Sall*-*Eco*RI fragment from pCMV-G. To generate pCHGP-2, the RRE (between positions 7754 and 8013 in HXB-2 [33]) was amplified by PCR from pV653RSN (31) by using the oligonucleotides RRE5 (5'GCAAGCTTCTGCAGAGCAGT GGGAAATAGG3') and RRE3 (5'GCAAGCTTACCCCAAATCCCCCAGGAG CTG3') and cloned immediately after the *gag-pol* gene in pCHGP-1. To generate pCHGP-3, the 0.65-kb *Stu*I-*Hind*III fragment from pCCAT-1 (44) was cloned after the *gag-pol* gene in pCHGP-1. To generate pCHGP-4, the MPMV CTE (between positions 8020 and 8240 in MPMV [40]) was amplified by PCR from pGem7 fz(-) MPMV(8001-8240) (6) and cloned behind the *gag-pol* gene in pCHGP-1.

To generate pV653CMV $\beta$ -gal, a CMV  $\beta$ -galactosidase ( $\beta$ -gal) cassette was first constructed by ligating the 0.75-kb *Xba*I-*Sall* fragment containing the CMV promoter from pCMV-G to the 3.1-kb *Xba*I-*Sma*I fragment containing the  $\beta$ -gal gene from pSP6- $\beta$ -gal (32) with pBluescript SK(-) (Stratagene, La Jolla, Calif.) to generate pCMV $\beta$ -gal. The 3.8-kb *Not*I-*Sma*I fragment containing the CMV  $\beta$ -gal cassette from pCMV $\beta$ -gal was ligated with the 8-kb fragment from *Bam*HI-digested pV653RSN. To generate pCMV-Tat, pCMV-G was digested with *Xba*I, and the 4.7-kb fragment containing the CMV promoter was ligated with the 0.36-kb *Sall*-*Bam*HI fragment containing the Tat-coding region from pCV1 (4).

To generate pCMV-env, the 2.7-kb *Xba*I fragment from pCMVEnv-amDra containing the amphotropic envelope coding region was ligated with the 4.7-kb *Bam*HI fragment from pCMV-G. The construction of pCMV-G has been described previously (45). pCMV-*rev* was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program.

Cells. HeLa, HT1080, and 293T cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (FCS). SupT1 cells were maintained in RPMI 1640 medium supplemented with 10% FCS. Primary human fibroblast CCD 1059sk cells (obtained from the American Type Culture Collection; no. CRL2072) were grown in Eagle's minimum essential medium supplemented with 10% FCS. Quiescent CCD 1059sk cells were obtained by growing confluent cells for 5 to 10 days in minimum essential medium with 10% FCS, resulting in a population of cells in growth arrest at the G<sub>0</sub>/G<sub>1</sub> phase as determined by flow cytometry analysis. Quiescent HeLa cells were obtained by plating 2  $\times$  10<sup>5</sup> cells in each well of a 12-well plate 24 h prior to gamma irradiation. The cells were subjected to gamma irradiation at a dose of 4,000 rads, and approximately 90% of the cells were arrested at G<sub>2</sub> phase 3 days after irradiation as determined by flow cytometry analysis.

**Vector production and infection of target cells.** To generate infectious HIV vectors, 293T cells were seeded at a density of 4  $\times$  10<sup>6</sup> cells per 10-cm-diameter culture dish. The infectious vector with all of the accessory proteins was generated by cotransfecting 10  $\mu$ g of pCMV-HIV-1, 10  $\mu$ g of pCMV-G, and 20  $\mu$ g of pV653CMV $\beta$ -gal by the calcium phosphate coprecipitation method (16). The culture medium was replaced 6 to 8 h later, and the culture supernatant was collected 18 h after transfection, filtered through 0.45- $\mu$ m-pore-size filters, and stored at -80°C. To generate the vector without any accessory protein, 293T cells were cotransfected with 8  $\mu$ g of pCHGP plasmid series, 8  $\mu$ g of pCMV-G, 16  $\mu$ g of pV653CMV $\beta$ -gal, 4  $\mu$ g of pCMV-Tat, and 4  $\mu$ g of pCMV-Rev. 293GP/LCZL cells containing an MLV-based provirus with the CMV  $\beta$ -gal cassette were used in this study. The VSV G protein-pseudotyped MLV vector was generated as described before (45).

To determine the vector titer, 5  $\times$  10<sup>4</sup> HT1080 cells were plated in a 12-well plate in the presence of 8  $\mu$ g of Polybrene per ml 24 h prior to infection. The cells were infected overnight with various dilutions of the vector and assayed for  $\beta$ -gal activity 48 h after infection.

To assay for  $\beta$ -gal activity, cells were washed once with phosphate-buffered saline, fixed in 1.25% glutaraldehyde for 15 min, and stained for 4 h at 37°C in a solution containing 5 mM potassium ferriferrocyanide, 400  $\mu$ g of X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside) (GBT, St. Louis, Mo.) per ml, and 1 mM MgCl<sub>2</sub>.

**Helper virus assays.** To detect helper virus, SupT1 cells were infected with 6  $\times$  10<sup>6</sup> infectious vector particles containing all accessory proteins or 6  $\times$  10<sup>5</sup> vector particles containing no accessory proteins in the presence of 8  $\mu$ g of Polybrene per ml in T75 flasks. Passage of the infected cells was allowed to continue for 10 weeks. During each passage, the culture supernatant was harvested and stored at -80°C. The p24 level in the supernatant was determined by an enzyme-linked immunosorbent assay (Coulter Corporation, Miami, Fla.). No replication-competent virus was detected after 10 weeks of culture.

#### RESULTS

**Effect of accessory proteins on vector production.** To generate an infectious HIV-1-based vector containing all accessory proteins, 293T cells were cotransfected with the three constructs shown in Fig. 1A. As shown in Table 1, the three-plasmid cotransfection resulted in the production of a vector titer of 3.9  $\times$  10<sup>6</sup> infectious units (IU)/ml. To determine whether the accessory proteins have any effect on vector production, the gene encoding each of the four accessory proteins in pCMV-HIV-1 was mutated. In addition, a combination of both Vpr and Nef mutations was introduced into pCMV-HIV-1. As shown in Table 1, mutations in the accessory proteins had very little effect on vector production from transiently transfected 293T cells. After 10 weeks of culture of cells transduced with high-titer vector, no helper virus could be detected in any experiment by screening for p24 expression.

**Effect of RNA transport elements on vector production.** To address the question of whether other RNA transport elements can substitute for the RRE and Rev function, a series of pCHGP plasmids were constructed (Fig. 1B). pCHGP-1 contains the HIV-1 *gag* and *pol* genes under the control of the CMV promoter. The HIV RRE was inserted immediately downstream of the *gag-pol* gene to generate pCHGP-2 to facilitate the *gag-pol* RNA exit from the nucleus. In pCHGP-3 and pCHGP-4, the posttranscriptional regulatory element

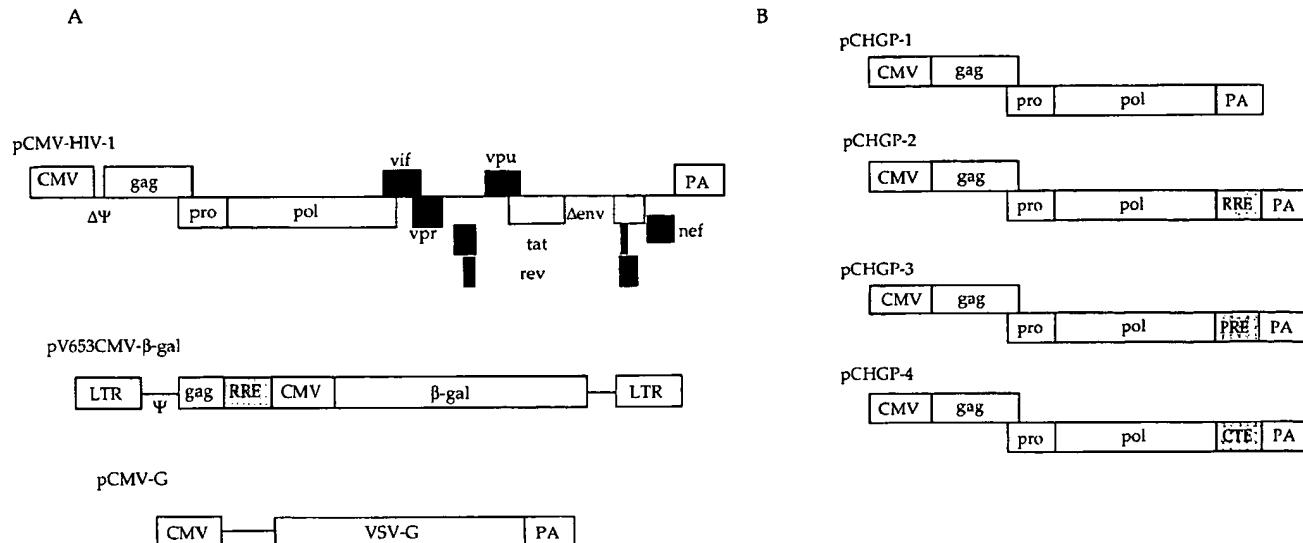


FIG. 1. Structures of expression plasmids of the HIV-1-based vector production system. (A) Packaging system; (B) minimal *gag-pol* constructs. Expression of packaging and VSV G protein (VSV-G) envelope constructs is driven by the CMV immediate-early promoter (CMV). The polyadenylation signal (PA) is derived from the rabbit  $\beta$ -globin gene. pCMV-HIV-1 contains all the HIV-1 genes except a 19-bp deletion in the packaging signal ( $\Delta\Psi$ ) and a 600-bp deletion in the *env* open reading frame ( $\Delta\text{env}$ ). Accessory-protein open reading frames are shown as solid boxes. The RNA transport elements are presented as shaded boxes. The vector genome pV653CMV- $\beta$ -gal consists of the HIV-1 LTRs flanking the RRE and the reporter gene ( $\beta$ -gal) driven by the CMV promoter.

(PRE) from HBV and the CTE from MPMV, respectively, were inserted immediately downstream of the *gag-pol* gene.

To test whether these constructs produce HIV *gag-pol* particles, they were cotransfected into 293T cells with or without pCMV-Rev, and the p24 level in the culture supernatant was determined 48 h after transfection. As shown in Table 2, pCHGP-1 generates a low level of p24 with or without Rev, consistent with the observation that the RRE is required for efficient transportation of the HIV *gag-pol* transcript from the nucleus into the cytoplasm. The presence of the RRE in pCHGP-2 results in 30-fold stimulation of p24 production when Rev is coexpressed. The HBV PRE transport element did not have any stimulating effect on p24 expression; however, in the presence of Rev, a 3.5-fold increase in the p24 level relative to that for pCHGP-1 was observed. Finally, the presence of the CTE from MPMV increases the p24 level seven- to ninefold relative to that of pCHGP-1-transfected cells. As expected, the stimulation of p24 production by the CTE is not Rev dependent. Therefore, the CTE is able, to some extent, to substitute for the Rev and RRE requirement in our system.

To test whether these packaging plasmids generate infectious vectors in the absence of accessory protein, each of the constructs from the pCHGP series was cotransfected with pCMV-G, pV653CMV- $\beta$ -gal, and pCMV-Tat, which is required for the efficient expression of the genomic RNA derived from pV653CMV- $\beta$ -gal. In addition, pCMV-Rev was either included or not included in each cotransfection experiment to determine the effect of Rev on the vector titer. Vector particles generated 24 to 48 h after transfection were harvested, and titers were determined in HT1080 cells by X-Gal staining and counting of positive blue cells.

As shown in Table 3, pCMV-HIV-1 generated a titer of about  $10^7$  IU/ml, and the titer was not affected by cotransfection of pCMV-Rev. Cells transfected with pCHGP-1 generated low titers with or without Rev. In contrast, cells cotransfected with pCHGP-2 and pCMV-Rev generated a titer which was at least 3 orders of magnitude higher than that without pCMV-Rev. For pCHGP-3, a very low vector titer was obtained from

the transfected cells, consistent with the low p24 level derived from pCHGP-3-transfected cells (Table 2). Despite the small effect of Rev on the p24 level generated from pCHGP-4-transfected cells (Table 2), the vector titer derived from this construct increased more than 50-fold with cotransfection of pCMV-Rev. This increase in titer with Rev probably reflects the fact that Rev stimulates the transportation of the vector genomic RNA derived from pV653CMV- $\beta$ -gal into the cytoplasm, thereby allowing more viral RNA to be packaged into virions. Overall, the vector titer in Table 3 corresponds to the p24 level generated by each packaging construct shown in Table 2. These results demonstrate that the CTE from MPMV can substitute for the function of RRE and Rev to facilitate HIV gene expression, whereas the PRE from HBV fails to perform this function. However, a combination of the RRE and Rev is able to generate a vector titer which is at least 10-fold higher than that generated by the CTE (compare vector titers of pCHGP-2 and pCHGP-4 in Table 3).

**Sodium butyrate stimulates vector production from 293T cells.** Sodium butyrate has been shown to stimulate the activity of the HIV-1 LTR and the CMV immediate-early promoter (24, 39). To determine whether sodium butyrate had any effect on vector production, vector particles were generated from pV653CMV- $\beta$ -gal in the presence of various concentrations of sodium butyrate. As shown in Fig. 2, addition of sodium bu-

TABLE 1. Effect of HIV-1 accessory proteins on vector production

pCMV-HIV	Titer (IU/ml) <sup>a</sup>
Wild type	$(3.9 \pm 0.7) \times 10^6$
vpr(-)	$(8.3 \pm 2.0) \times 10^6$
vpu(-)	$(4.8 \pm 1.0) \times 10^6$
vif(-)	$(6.0 \pm 0.7) \times 10^6$
nef(-)	$(1.1 \pm 0.1) \times 10^7$
nef(-)vpr(-)	$(3.1 \pm 0.3) \times 10^6$

<sup>a</sup> The vectors were harvested 24 h after transfection, and the titer was determined in HT1080 cells by counting of blue cells after X-Gal staining. The numbers are the averages from triplicate experiments  $\pm$  standard deviations.

TABLE 2. Expression of p24 in pCHGP-transfected cells<sup>a</sup>

Plasmid construct	pCMV-Rev	p24 (ng/ml) <sup>b</sup>
pCHGP-1	—	1.4 ± 0.2
	+	1.8 ± 0.1
pCHGP-2	—	3.5 ± 0.2
	+	98.8 ± 14.6
pCHGP-3	—	0.4 ± 0.1
	+	6.2 ± 0.7
pCHGP-4	—	10.2 ± 0.8
	+	16.4 ± 1.0
pCMV-HIV-1	—	6,958 ± 349
	+	7,471 ± 287

<sup>a</sup> 293T cells were transfected in a 10-cm-diameter culture dish with 20 µg of pCHGP as indicated and 10 µg of either pCMV-Rev (+) or pBluescript (—). Forty-eight hours after transfection, the culture medium was assayed for p24 protein expression.

<sup>b</sup> Averages from triplicate experiments ± standard deviations.

tyrate had little effect on the vector titer generated from pCMV-HIV-1-transfected cells, whereas the titer generated from pCHGP-2-transfected cells increased approximately 15-fold in the presence of 4 mM sodium butyrate. To determine the possible reason for the titer increase, the p24 level in the culture supernatant of transfected 293T cells was determined. The p24 level of the pCMV-HIV-1-transfected cells increased slightly, from 6.6 µg/ml, with a titer of  $6.0 \times 10^6$  IU/ml, in the absence of sodium butyrate to 9.9 µg/ml, with a titer of  $1.1 \times 10^7$  IU/ml, in the presence of 4 mM sodium butyrate. However, the p24 level of the pCHGP-2 transfected cells increased from 0.1 µg/ml, with a titer of  $4.4 \times 10^5$  IU/ml, in the absence of sodium butyrate to 1.1 µg/ml, with a titer of  $6.4 \times 10^6$  IU/ml, in the presence of 4 mM sodium butyrate. Thus, the 10-fold increase in p24 production in pCHGP-2-transfected cells in the presence of sodium butyrate can account for the observed 15-fold increase in the vector titer shown in Fig. 2. The lack of stimulation in pCMV-HIV-1-transfected cells may reflect the extremely high level of p24 already generated by this construct in the absence of sodium butyrate.

**Effect of accessory proteins on vector infectivity.** To study the ability of the HIV vector to infect quiescent cells and the effect of the accessory proteins on infectivity, HeLa cells were exposed to gamma irradiation to arrest cells at the G<sub>2</sub> phase of the cell cycle. Proliferating or growth-arrested HeLa cells were transduced with either MLV-β-gal, a β-gal gene-containing MLV vector; the HIV-1-based vector v653CMVβ-gal(+), containing all four accessory proteins; or v653CMVβ-gal(—), containing no accessory protein. Positive cells were scored by X-Gal staining 2 days after transduction. Results for HeLa cells were expressed as the percentages of titers observed with the same virus preparations in growing HT1080 cells. As shown in Fig. 3, no significant difference in titer was observed in proliferating or quiescent HeLa cells transduced with either the v653CMVβ-gal(+) or the v653CMVβ-gal(—) vector. In contrast, the transduction efficiency of the MLV vector in quiescent cells was reduced more than 2,000-fold. Similar results were obtained with irradiated HT1080 cells transduced with the three vectors (data not shown). To demonstrate that the observed β-gal activity is not due to pseudotransduction of the β-gal activity present in the vector preparation, proliferating HeLa cells transduced with the vector were treated with increasing concentrations of 3'-azido-3'-deoxythymidine. Both

TABLE 3. Vector production from pCHGP-transfected 293T cells

Plasmid construct	pCMV-Rev	Titer (IU/ml) <sup>a</sup>
pCHGP-1	—	(1.4 ± 0.3) × 10 <sup>2</sup>
	+	(2.5 ± 1.1) × 10 <sup>2</sup>
pCHGP-2	—	(1.0 ± 0.6) × 10 <sup>2</sup>
	+	(6.0 ± 0.6) × 10 <sup>5</sup>
pCHGP-3	—	(1.1 ± 0.4) × 10 <sup>1</sup>
	+	(3.6 ± 0.2) × 10 <sup>2</sup>
pCHGP-4	—	(8.4 ± 1.2) × 10 <sup>2</sup>
	+	(5.3 ± 1.7) × 10 <sup>4</sup>
pCMV-HIV-1	—	(1.2 ± 0.4) × 10 <sup>7</sup>
	+	(1.1 ± 0.1) × 10 <sup>7</sup>

<sup>a</sup> The infectious vector was generated as described in Materials and Methods. Titers were determined in HT1080 cells. The numbers are the averages from triplicate experiments ± standard deviations.

the number of blue cells and the β-gal activity in cell extracts decreased with increasing concentrations of 3'-azido-3'-deoxythymidine (data not shown). These results demonstrate that, in contrast to the MLV vector, HIV-1-based vectors can transduce quiescent cells efficiently and the HIV-1-encoded accessory proteins are not required to transduce these cells.

To test the infectivity of HIV-1-based vectors in other cell types, primary human skin fibroblasts were allowed either to proliferate or to grow to confluence and then were infected with the three retroviral vectors described above. Fibroblasts grown to confluence become contact inhibited and arrested in the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle (data not shown). As shown in Fig. 4, the three vectors exhibit similar transduction efficiencies in dividing fibroblasts. However, in quiescent cells, MLV-β-gal vector transduction dropped to barely detectable levels. The capacity of v653CMVβ-gal(+) remained unchanged. In contrast, v653CMVβ-gal(—), which is defective for the HIV-1 accessory proteins, showed a four- to sevenfold-decreased level of efficiency in transducing the contact-inhibited fibroblasts relative to the v653CMVβ-gal(+) vector. These results suggest that the requirement for accessory proteins for efficient transduction by HIV-1-based vectors is cell type dependent.

## DISCUSSION

The ability of HIV-based vectors to infect terminally differentiated cells and quiescent cells makes them suitable vectors for direct *in vivo* gene delivery. However, large-scale production and purification of HIV vectors require the establishment of stable packaging cell lines. Since HIV-1 encodes at least nine proteins and stable expression of some of these proteins, such as Vpr, is known to be extremely toxic to the cells (21, 34), selective expression of only those proteins absolutely essential for vector production and infectivity is important.

Consistent with previous studies, transient transfection into 293T cells of all the required components for HIV production resulted in high-titer vector production in the present study. Mutation of the genes encoding HIV-1 accessory proteins has little effect on vector production. Efficient HIV-1 infection requires the presence of the Nef protein, which appears to facilitate virus capsid disassembly upon infection (3, 38). The requirement for Nef for virus uncoating can be bypassed if the virus is pseudotyped with VSV G protein (2), which may explain why there was no significant titer reduction in the present study. Vpu has been shown to facilitate the release of budding

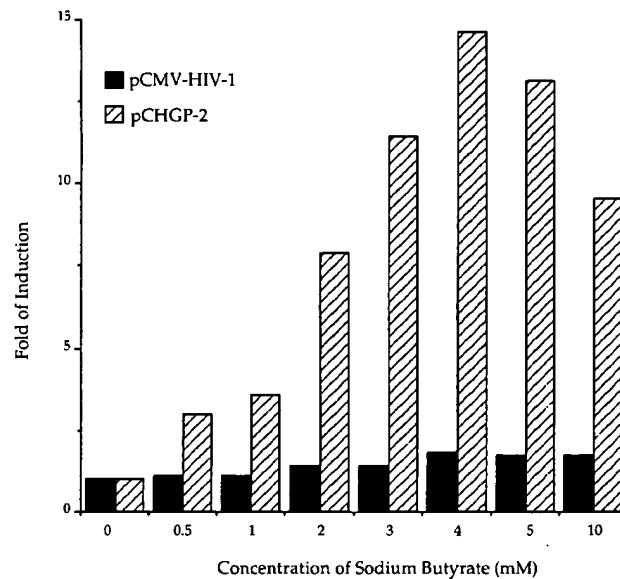


FIG. 2. Stimulation of vector production by sodium butyrate. Vectors derived from either pCMV-HIV-1 or pCHGP-2 were generated in 293T cells in the presence of various concentrations of sodium butyrate as indicated. Titers were determined in HT1080 cells as described in Materials and Methods. Values are the ratios of titers with sodium butyrate to titers without sodium butyrate for each vector. This experiment was repeated once, and similar results were obtained (data not shown).

virus particles from the surfaces of infected cells (42). The enhancement of capsid release is cell type dependent and is not limited to HIV; it can also facilitate the release of visna virus and MLV from infected cells (15, 37). The Vpu-deficient vector is generated from 293T cells by transient transfection. Since 293T has an extremely high transfection efficiency, this procedure presumably produces excessive amounts of the vector

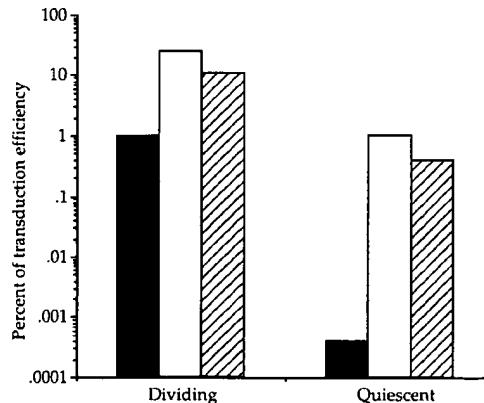


FIG. 3. Transduction efficiency in HeLa cells. Three hundred microliters of MLV-β-gal with a titer of  $2.8 \times 10^6$  IU/ml (■), v653CMVβ-gal(-) with a titer of  $1.4 \times 10^6$  IU/ml (▨), or v653CMVβ-gal(+) with a titer of  $3.7 \times 10^6$  IU/ml (□) was used to transduce actively dividing or growth-arrested HeLa cells in 12-well plates. The cells were harvested 2 days after transduction, and the total β-gal activity was determined by blue-cell count after X-Gal staining. The results are presented as the percentage of the vector titer observed in HT1080 cells for each viral preparation ([titer in dividing or quiescent HeLa cells/titer in HT1080 cells]  $\times 100$ ). The experiment was repeated with 100 and 30 μl of the same vector stocks, and similar results were observed. Transduction of the HeLa cells with different vector stocks was repeated at least twice, and similar results were obtained (data not shown).

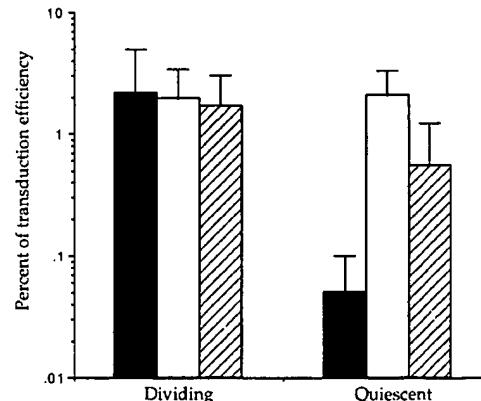


FIG. 4. Transduction efficiency in human skin fibroblasts. Ten microliters of MLV-β-gal (■), v653CMVβ-gal(-) (▨), or v653CMVβ-gal(+) (□) was used to infect dividing and quiescent fibroblasts in a 12-well plate. Two days after transduction, the titer was determined by blue-cell counting after X-Gal staining. Data for transduction of quiescent and dividing fibroblasts are presented as the percentage of the titer observed in growing HT1080 cells for each viral preparation ([titer in growing or quiescent fibroblasts/titer in HT1080 cells]  $\times 100$ ). The values are averages from four experiments, with standard deviations.

genome as well as HIV-encoded proteins, which may make the Vpu effect negligible. Alternatively, Vpu may have no effect on virus release from 293T cells, as observed for a number of other cell lines (36). The Vpr protein, like HIV MA and integrase, contains a nucleophilic determinant that permits nuclear localization of viral nuclear capsid and replication in nondividing cells (18). Mutation of Vpr, however, has no effect on the virus infectivity in proliferating cells such as the growing HT1080 cells used to determine the titer of the Vpr-deficient vector. Vif acts during virus assembly to make the virus particle competent for subsequent infection (43). However, this effect is dependent on the cell type from which the virus is generated (10). The absence of a titer decrease for the Vif-deficient vector may reflect the permissiveness of 293T cells to complement the Vif defect.

When the minimal pCHGP-2 packaging construct is used, our results showed, as predicted, that p24 expression from transfected cells is strongly dependent on the presence of Rev. Interestingly, when the HBV PRE transport element was used, its mRNA-transporting activity appeared to be strongly increased when the Rev protein was coexpressed, although it was still considerably lower than that of the RRE-Rev system. This transactivating property was not observed when the RevM10 (27) transdominant negative mutant was used (14). In addition, it has been suggested that HBV and RRE mRNA transport from the nucleus to the cytoplasm could involve the same pathway (35). Together, these data suggest that Rev could enhance PRE transport activity directly by binding the PRE sequence or indirectly by recruiting cellular factors involved in the nuclear export mechanism. Insertion of the HBV PRE into the second intron of HIV-1 has been shown to increase the accumulation of the unspliced RNA in the cytoplasm, demonstrating that this element can facilitate RNA exit from the nuclei (19). However, the present study demonstrates that the PRE from HBV slightly increases p24 expression or vector production from the transfected cells only when Rev is expressed. Therefore, PRE, as a transport element, does not appear to be useful for our purpose to bypass the HIV-1 Rev protein requirement.

In contrast, pCHGP-4, which contains the CTE derived from the MPMV genome, enables Rev-independent expres-

sion of p24, as it is not stimulated significantly by cotransfection of pCMV-Rev. However, the p24 level derived from cells transfected with pCHGP-4 is at least fivefold lower than that with pCHGP-2 in the presence of Rev. In these experiments, pCHGP-2 was cotransfected with pCMV-Rev, and as a result, large amounts of Rev may be available in the transfected cells to efficiently transport RRE-containing transcripts from nuclei into the cytoplasm. In contrast, the CTE in pCHGP-4 interacts with endogenous factors which may be in limited supply compared with Rev generated from transient transfection. One such factor interacting with the CTE of MPMV has recently been identified as ATP-dependent RNA helicase A (41). Thus, a difference in the endogenous levels of the protein factors required for efficient transportation of either RRE- or CTE-containing transcripts may account for the observed difference in p24 expression of these two constructs. Alternatively, the RRE and Rev may be intrinsically more efficient than the CTE to transport the HIV-encoded messages. The efficiency of transporting HIV-encoded messages by either the RRE or CTE can be elucidated with cell lines stably expressing the HIV Rev protein.

Our results demonstrate that HIV-derived vectors transduce nonproliferating cells efficiently, whereas the MLV vector fails to give detectable transduction. This observation is consistent with the fact that at least three nuclear localization signal-containing proteins, including MA, Vpr, and integrase, are present in the lentivirus particle, and these proteins facilitate active transport of the nucleocapsid from the cytoplasm into the nuclei of the infected cells. The functional redundancy of these proteins may explain why accessory protein-deficient vector particles can still infect growth-arrested HeLa cells at the same efficiency as vector particles containing all of the accessory proteins. These results are consistent with the recent observations reported by others that the accessory proteins are not required for efficient infection of growth-arrested 293T cells (23, 46). However, the infectivity of the accessory protein-deficient vector in contact-inhibited primary human skin fibroblasts is reduced approximately threefold compared with that of the vector containing all of the accessory proteins. Our results suggest that for efficient infection of this cell type, the accessory proteins, either alone or in combination, are beneficial. We conclude that the effect of HIV accessory proteins on infectivity is dependent on the cell type or cell proliferation state. This conclusion is in agreement with the results reported by Zufferey et al. (46) and Kafri et al. (22) that for efficient infection of human macrophages in culture and adult mouse livers *in vivo*, the presence of accessory proteins enhances infectivity, whereas they are dispensable for efficient infection of neuronal cells *in vivo*.

High-titer HIV-1 vectors have so far been generated from 293T cells by transient transfection. Although this method is convenient, it is not suitable for mass vector production for clinical application. Establishment of stable packaging cell lines for HIV vectors not only overcomes the mass production problem, but it will also make helper virus generation unlikely because multiple homologous recombination events are required for such an event to occur. Such events occur more frequently before stable transfection and integration take place. Our results suggest that while the CTE can partially alleviate the requirement for stable Rev expression in the packaging cell lines, a combination of Rev and the RRE still generates significantly higher vector titers than the CTE. The HIV-1-encoded accessory proteins are not required for efficient vector production from 293T cells, and the infectivity of the resulting vector is similar to that of the vector containing all accessory proteins except in the case of primary human skin

fibroblasts. Recent results from Kim et al. (23) suggest that the requirement for Tat can be bypassed by using an HIV vector containing a hybrid HIV LTR with the U3 regions replaced with the CMV promoter. Based on these studies, the indispensable components of the packaging cell lines for HIV vectors should therefore be only *gag*, *pol*, Rev, and VSV G protein. To efficiently infect cell types such as quiescent skin fibroblasts and hepatocytes, some of the accessory proteins will have to be expressed in the packaging cell lines. The present study should help to facilitate the successful establishment of packaging cell lines for HIV-1-derived vectors.

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## A Packaging Cell Line for Lentivirus Vectors

TAL KAFRI, HENRIETTE VAN PRAAG, LING OUYANG,  
FRED H. GAGE, AND INDER M. VERMA\*

*Laboratory of Genetics, The Salk Institute, La Jolla, California 92037*

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**Lentivirus vectors can transduce dividing and nondividing cells. Using three-plasmid transient transfections, high-titer ( $>10^9$  IU/ml) recombinant lentivirus vectors pseudotyped with vesicular stomatitis virus G (VSV-G) protein can be generated (T. Kafri et al., *Nat. Genet.* 17:314-317, 1997; H. Miyoshi et al., *Proc. Natl. Acad. Sci. USA* 94:10319-10323, 1997; L. Naldini et al., *Science* 272:263-267, 1996). The recombinant lentiviruses can efficiently infect brain, liver, muscle, and retinal tissue *in vivo*. Furthermore, the transduced tissues demonstrated long-term expression of reporter genes in immunocompetent rodents. We now report the generation of a tetracycline-inducible VSV-G pseudotyped lentivirus packaging cell line which can generate virus particles at titers greater than  $10^6$  IU/ml for at least 3 to 4 days. The vector produced by the inducible cell line can be concentrated to titers of  $10^9$  IU/ml and can efficiently transduce nondividing cells *in vitro* and *in vivo*. The availability of a lentivirus packaging cell line will significantly facilitate the production of high-titer lentivirus vectors for gene therapy and study of human immunodeficiency virus biology.**

Retrovirus vectors have been used extensively for gene therapy (23). However, currently available recombinant retroviral vectors are not suitable for *in vivo* gene delivery because they can transduce dividing cells only. The advent of lentivirus vectors has overcome this obstacle and can efficiently transduce a variety of tissues *in vivo*, including brain, liver, muscle, retina, and hematopoietic cells. Sustained expression of the transgene in the transduced tissues of immunocompetent mice or rats further boosts their utility as a desirable vector for gene therapy.

Human immunodeficiency virus (HIV)-based vectors have been generated by transient transfection of (i) the packaging construct plasmid encoding Gag-Pol, Tat, Rev, Nef, Vpr, Vpu, and Vif proteins; (ii) a plasmid encoding vesicular stomatitis virus G (VSV-G) protein; and (iii) the vector containing the HIV long terminal repeats (LTRs), packaging signal, Rev response element, and foreign promoter driving the transgene (10, 13-15). Recombinant viruses with titers of  $3 \times 10^6$  IU/ml were generated which could be concentrated by centrifugation to titers of  $1 \times 10^9$  to  $3 \times 10^9$  IU/ml (10, 12, 13). To facilitate vector production for *in vivo* experiments in larger animals we need to develop a stable VSV-G pseudotyped lentivirus vector packaging cell line. An earlier report on the development of a tetracycline-inducible VSV-G pseudotyped murine leukemia virus vector packaging cell line encouraged us to adopt a similar approach (16). Lentivirus vector packaging cells have previously been described but the vector titers were quite low (1, 3, 6, 18, 19, 25). Additionally, some of the viral accessory proteins in these cell lines were either missing or nonfunctional (25). In some of these cell lines, the gene encoding the envelope protein and the genes encoding the packaging proteins were transcribed from the same expression cassette, thereby increasing the probability of generation of helper viruses. Furthermore, none of the earlier cell lines expressed the VSV-G envelope protein that allows vector concentration and expands the range of target tissues. A major hurdle in the construction of a packaging cell line generating recombinant lentiviruses

was that VSV-G and some HIV proteins, including protease and Vpr, have been reported to be cytotoxic (11, 17, 20). Here we describe the generation of an inducible lentivirus vector packaging cell line that was constructed in 293 cells and that constitutively expresses a tetracycline-regulated transactivator (tTA) (8, 9). All HIV type 1 (HIV-1) genes (excluding the HIV-1 envelope gp 120) are transcribed in the cell line from a single expression cassette, which is regulated by tetracycline (8). In addition, the cell line expresses the VSV-G envelope protein and the green fluorescent protein (GFP) from a bidirectional tetracycline-regulated promoter. This gives the advantage not only of pseudotyping the lentivirus vectors with the VSV-G envelope but also of monitoring the induction process. A rapid and high level of gene induction can be obtained by the addition of sodium butyrate. Following transduction by lentivirus vectors, the novel packaging cell line can generate vector particles at titers greater than  $10^6$  IU/ml for at least 3 to 4 days. The vectors produced by the inducible cell line can be concentrated and can transduce growth-arrested cells in culture and in terminally differentiated neurons in immunocompetent rats.

### MATERIALS AND METHODS

**Plasmid construction.** pSKVG was constructed by cloning the *Eco*RI VSV-G (Indiana serotype) from pMDG (15) into the *Eco*RI site of Bluescript SK<sup>+</sup>. The GFP coding fragment was excised from pEGFP-N1 by *Sac*I-*Not*I digestion and ligated to the *Sac*I-*Not*I fragment of Bluescript SK<sup>+</sup> to create pSKGFP. The *Pst*I fragment containing the GFP coding region from pSKGFP was ligated to the *Pst*I site in pBI (Clontech 6152-1) to create pBIGF. The *Nhe*I-*Eco*RV fragment containing the VSV-G coding region from pSKVG was ligated to the *Xba*I-*Pvu*II sites in pBIGF to create pBIGFVS. The *Bam*HI-*Bgl*II fragment containing a minimal cytomegalovirus (CMV) immediate-early gene promoter linked to seven tandem copies of the tetR-binding site replaced the CMV promoter (*Bgl*II-*Bam*HI fragment) in pcDNAneo to create phCMVn. The plasmid pPTK was constructed by ligation of the *Bgl*I-*Sac*II fragment bearing the genes encoding all the HIV-1 proteins from pΔR8.2 (14) to the *Bgl*I (partial)-*Sac*II fragment from phCMVn. The *Xba*I fragment containing the neomycin resistance gene was deleted from ptTet-Off (Clontech K1620-A) to create ptTAΔn, from which a fusion protein containing the carboxy terminus of the tetracycline repressor and the herpes simplex virus VP16 transactivation domain is expressed under the control of the CMV promoter. The pCLBFP was constructed by ligation of the *Bam*HI-*Eco*RI fragment containing the blue fluorescent protein (BFP) gene from pEBFP (Clontech 6068-1) into the *Bam*HI-*Eco*RI fragment of pCL. The *Hind*III-*Not*I fragment containing the BFP coding region was inserted into the *Hind*III-*Not*I fragment of pcDNA3.1/hygro (Invitrogen V870-20) to create pcDNABFP.

\* Corresponding author. Mailing address: Laboratory of Genetics, The Salk Institute, 10010 N. Torrey Pines Rd., La Jolla, CA 92037. Phone: (619) 453-4100. Fax: (619) 558-7454. E-mail: verma@salk.edu.

**Vector production.** All the cell lines in this study were maintained in Dulbecco's modified Eagle's medium containing tetracycline-free 10% fetal calf serum (Clontech 8630-1). To generate the stable cell line SODk0 that expresses the fusion protein tetracycline repressor-VP16 transactivation domain, human 293 embryonic kidney cells were cotransfected with 20  $\mu$ g of pTAA $\Delta$ n and 1  $\mu$ g of pSR $\alpha$ BSR that expresses the blastocidin resistance gene by the calcium phosphate precipitation method (5). Individual cell colonies were selected with 20  $\mu$ g of blastocidin/ml. The lentivirus vector stable packaging cell line SODk1 was generated by transfection of SODk0 cells with 10  $\mu$ g of pPTK and 10  $\mu$ g of pBIGFVS. The transfected cells were selected for neomycin resistance (400  $\mu$ g/ml) in the presence of 0.7  $\mu$ g of doxycycline/ml. Individual colonies were screened for HIV-1 p24 and GFP production and cell fusion in the presence or absence of doxycycline as follows. Cells from a confluent 10-cm plate were split by a ratio of 1:4 into PolyLysine precoated plates. Induced cells were cultured in the absence of doxycycline. Cell media were changed daily. Control cells were cultured in the presence of 0.7  $\mu$ g of doxycycline/ml. On day 4 postinduction, the levels of HIV-1 p24 in conditioned media were measured by enzyme-linked immunosorbent assay (ELISA) (DuPont). Cell fusion was used as a marker for VSV-G production, and GFP production was determined by fluorescence microscopy. The colony that was found to be negative for p24 and GFP production in the presence of doxycycline and yet showed the highest levels of p24 production (>800 ng/ml) with more than 85% green cells upon induction was chosen as the packaging cell line for the lentivirus vector. The lentivirus vector producer cell line SODk1CGFI was generated by transducing SODk1 cells with HRcmvGFP lentivirus vector using a multiplicity of infection (MOI) of 2.

SODk1CGFI cells were split from a confluent 10-cm plate into a precoated PolyLysine plate at a ratio of 1:4 in the absence of doxycycline. Twenty-four hours after the split, the cells were washed twice with phosphate-buffered saline (PBS) and refed with doxycycline-free media that contained 5 mM sodium butyrate. The media were replenished daily. Induced SODk1CGFI-conditioned media were filtered through a 0.45- $\mu$ m-pore-size filter and assayed daily for vector titers and p24 concentration by serial dilutions on 293 cells and by p24 ELISA, respectively. To further concentrate the vector, conditioned media were collected 3 days after addition of sodium butyrate filtered as described above and ultracentrifuged at 50,000  $\times$  g for 2 h. The pellet was resuspended and incubated for 2 h at 37°C in Tris-buffered saline (TBS) containing 10 mM MgCl<sub>2</sub>, four dNTPs (0.1 mM each), 3 mM spermine, and 0.3 mM spermidine. After a second ultracentrifugation at 50,000  $\times$  g for 2 h, the vector was resuspended in TBS with 2  $\mu$ g of Polybrene/ml. The concentrated vector was assayed for p24 concentration and titrated on 293 cells as described above.

**Protein analysis.** Induced (3 days after adding sodium butyrate) and uninduced (cultured in the presence of doxycycline) cells were lysed, and proteins were denatured by boiling for 10 min in a buffer containing 68 mM Tris (pH 6.8), 50 mM NaCl, 0.5 mM EDTA, 20  $\mu$ g of aprotinin/ml, 50  $\mu$ g of phenylmethylsulfonyl fluoride/ml, 1.5% sodium dodecyl sulfate (SDS), 5% glycerol, and 5%  $\beta$ -mercaptoethanol. Twenty micrograms of the denatured protein was separated on a 12.5% polyacrylamide gel containing SDS and blotted onto an Immobilon-P membrane (Millipore). After blocking with 5% nonfat milk in PBS-0.2% Tween 20 for 15 min, the membranes were incubated with mouse monoclonal anti-VSV-G (Sigma V-5507) or with rabbit HIV-1 Vpr (National Institute of Allergy and Infectious Diseases, AIDS Research and Reference Reagent Program, reagent 3252) and then with goat anti-mouse immunoglobulin horseradish peroxidase (Pierce) or donkey anti-rabbit horseradish peroxidase (Amersham), respectively. The protein bands were detected with an ECL kit (Amersham).

**Transduction of nondividing cells.** Serial dilution of induced SODk1CGFVGI-conditioned media was used to transduce either HeLa cells arrested by culturing in the presence of 15  $\mu$ g of aphidicolin/ml for 12 h or human embryo fibroblasts arrested by culturing for 48 h in 0.1% fetal calf serum. Titers were scored 48 h posttransduction by dividing the number of GFP-positive foci by the dilution factor.

**Assays for replication-competent virus.** We used three independent methods to assay for replication-competent virus.

(i) **tat transfer assay.** This assay is based on a reporter HeLa P4.2 cell line which expresses CD4 and contains an integrated lacZ reporter gene driven by the HIV LTR. Since the HIV LTR is not active in naïve HeLa cells, the expression of the lacZ gene in P4.2 cells can serve as a sensitive indicator for the presence of HIV tat (4). The sensitivity of the assay was of 20 tat-transducing units per ml of test medium. The use of the P4.2 cell line as an indicator of HIV-1 helper activity in lentivirus vector stocks was described extensively in the past (15). In our assay, 10<sup>5</sup> P4.2 cells were transduced with 10<sup>6</sup> IU of the vector. The transduced cells were serially passaged for 3 weeks (about five passages), after which they were scored for HIV-1 tat activity by X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside) staining. Using this method, no transduction of tat was detected.

(ii) **HIV gag transfer assay.** This method is based on measuring p24<sup>agg</sup> in conditioned media obtained from vector-transduced cells. The use of this method to assay for HIV-1 helper activity in lentivirus vector stocks and its sensitivity was previously described (15). In our assay, 10<sup>5</sup> P4.2 cells were transduced by vector particles at an MOI of 10. Following five passages in culture, the concentration of p24<sup>agg</sup> in conditioned media obtained from these cells was determined by ELISA (p24 ELISA kit; NEN Life Science Products). The detection limit of this method is  $\geq$ 1 pg/ml, which is about 1 to 2 IU/ml. By this method, vector

preparation is considered helper negative when p24 concentrations are below detection levels.

(iii) **Marker rescue assay.** A total of 10<sup>5</sup> P4.2 cells transduced with HR'CMV GFP lentivirus vector (p4.2G.) were plated on a 10-cm-diameter plate and transduced with 10<sup>6</sup> IU of the tester vector stock. The transduced cells were cultured for 3 weeks, after which conditioned medium was harvested and filtered through a 0.45- $\mu$ m-pore-size filter. A total of 10<sup>5</sup> 293T cells were transduced in a 10-cm plate with 10 ml of the medium in the presence of 4  $\mu$ g of Polybrene/ml. Seventy-two hours posttransduction, the cells were scored for GFP expression. Vector stock was considered helper free when no green cells were detected.

**FACS analysis.** Trypsinized cells were resuspended in 5 ml of culture medium and washed in PBS minus Ca<sup>2+</sup> and Mg<sup>2+</sup>. The cells were fixed in 3 ml of 5% paraformaldehyde, washed twice in PBS, and diluted in PBS to a concentration of 10<sup>6</sup> cells/ml. Fluorescence-activated cell sorter (FACS) analysis for cellular GFP and BFP was performed by FACScan analysis (Becton Dickinson) with the CellQuest program (version 3.0.1f; Becton Dickinson).

**RNA analysis.** Total cellular RNA was isolated by using a Qiagen RNeasy kit. RNA (8 mg) was electrophoresed on a 1.2% agarose-formaldehyde gel, transferred to Hybond-N<sup>+</sup> (Amersham) membrane, and hybridized at 68°C to a <sup>32</sup>P-labelled DNA probe. The membrane was washed twice for 15 min in buffer containing 0.1% SDS-2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 68°C and once in buffer containing 0.1% SDS-0.1× SSC at 68°C for 15 min. The washed membrane was exposed to Kodak X-Omat Blue XB-1 film.

**Transduction of brain of immunocompetent rat.** Adult female Fisher 344 rats ( $n = 6$ ) were anesthetized (80 mg of ketamine, 0.75 mg of acepromazine, and 4 mg of xylazine per ml/kg of body weight), and 2  $\mu$ l of the viral-vector concentrated stock (1  $\times$  10<sup>9</sup> IU/ml) was injected into the left striatum (anterior-posterior, +1; medial-lateral, 3.5; dorsal-ventral, 4) with a 10- $\mu$ l Hamilton syringe. After 4 weeks, the rats were deeply anesthetized and perfused intracardially with 100 ml of saline followed by 200 ml of 4% paraformaldehyde. Brains were postfixed for 24 h and stored in 30% phosphate-buffered sucrose at 4°C until sectioning. Brains were frozen and sectioned on a sliding microtome into 40- $\mu$ m slices. Sections were washed and blocked in TBS with 3% donkey serum and 0.3% Triton X-100 (TBS plus). Primary antibodies raised in two different species were pooled in TBS plus and incubated for 48 h at 4°C. The antibody for NeuN (mouse monoclonal antibody [a generous gift of R. J. Mullin]; 1:20) was combined with antibody for GFAP (glial fibrillary acidic protein [guinea pig polyclonal antibody [Advanced Immunochemicals]; 1:500]). Corresponding secondary antibodies (donkey anti-mouse Texas red and donkey anti-guinea pig Cy5; 1:250) were pooled, and sections were incubated for 4 h at room temperature following washing in TBS plus. Every 12th section was mounted and coverslipped with DABCO-PVA. Sections were analyzed by confocal scanning laser microscopy (Bio-Rad). To determine the nature of transduced cells, double-labelled sections were scanned with a confocal laser microscope, and a representative sample of 100 transduced cells was examined for colocalization of GFP with either NeuN or GFAP.

## RESULTS

**SODk0: a tTA cell line.** The extensive characterization of the tetracycline-inducible system encouraged us to use this regulatory control in the development of the lentivirus vector packaging cell line. As a first step, we generated a 293 (human embryonic kidney) cell line that constitutively expresses the tTA. The tTA is a fusion product of the amino-terminal-DNA binding domain of the tet repressor and the carboxy-terminal activation domain of VP-16 from herpes simplex virus (8). In the absence of tetracycline, tTA binds to the tet-responsive elements (TRE) in the tet operator (tetO) and efficiently activates transcription from downstream minimal promoters. The association between tTA and the TRE is prevented by tetracycline; therefore, in the presence of low concentrations of tetracycline or its derivative doxycycline, transcription from TRE is turned off. We cotransfected 293 cells with a plasmid, pTAA $\Delta$ n, that expresses tTA from the early promoter of CMV and with plasmid pSR $\alpha$ BSR expressing the blastocidin resistance gene from the SR $\alpha$  promoter (21). Stably transfected clones were selected by culturing in the presence of blastocidin, and 20 cell clones whose growth rates did not differ significantly from that of the parental 293 cells were screened for tTA expression. Each clone was cotransfected with plasmid pBIGF that expresses GFP from a tetracycline-regulated promoter and plasmid pcDNABFP expressing BFP from a CMV promoter. The transfected cells were cultured either in the presence or the absence of doxycycline, a potent derivative of

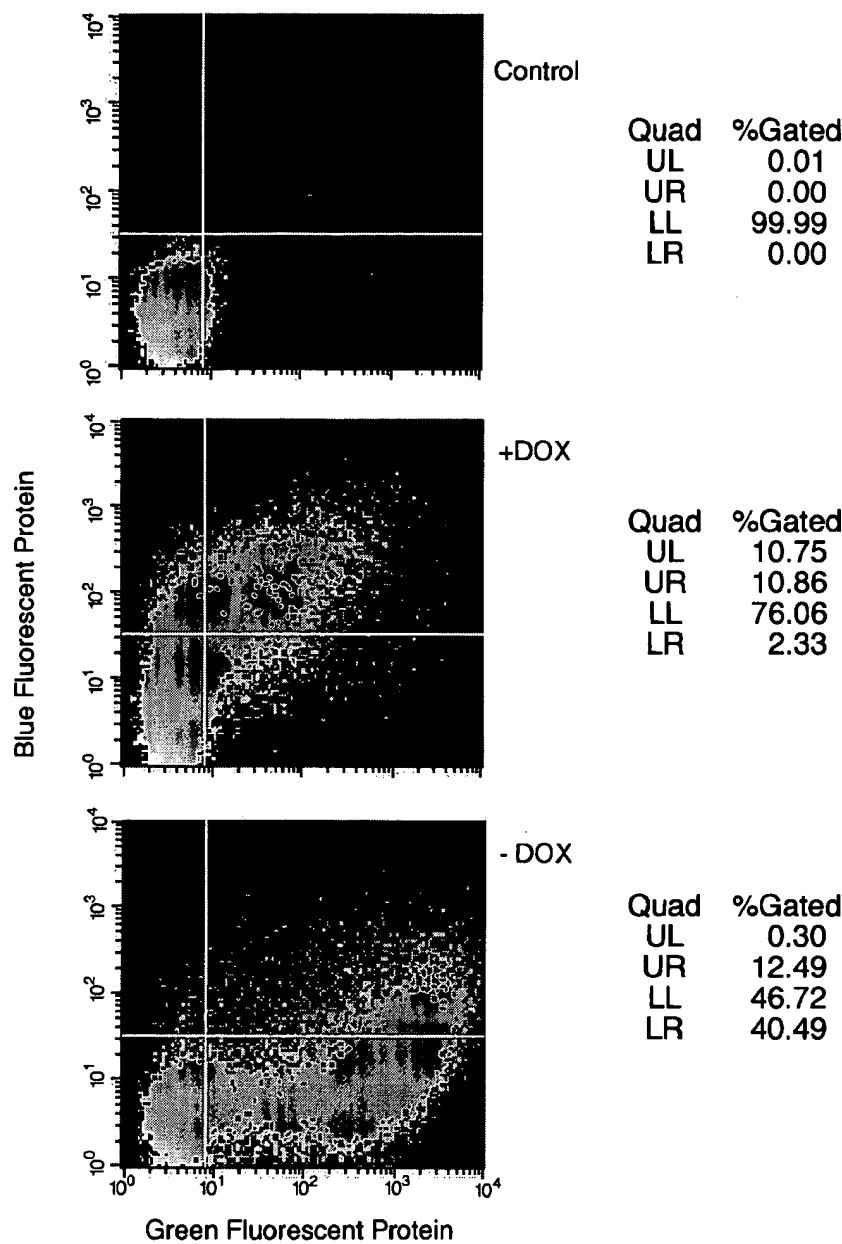


FIG. 1. Screening for tTA-expressing 293 cells. Human embryonic kidney 293 cell clones that stably expressed the tTA gene were transiently cotransfected with the pBIGF plasmid that produced the GFP under the control of the tetracycline-regulated promoter and the pcDNABFP plasmid that expressed the BFP under the control of the CMV promoter. Activation of the tetracycline-regulated promoter by the tTA in the presence and the absence of doxycycline (DOX) was evaluated by GFP expression and was determined by FACS analysis (x axis). BFP expression from the CMV promoter served as a standard for transfection efficiency and transcription activity and was determined by FACS analysis (y axis). Nontransfected human embryonic kidney 293 cells that expressed the tTA gene served as a negative control for either GFP or BFP expression.

tetracycline (9). By fluorescence microscopy and FACS analysis we could evaluate the relative expression of GFP (as a reporter for tTA activity) and the expression of BFP (as a standard for transfection efficiency and transcription activity). The FACS analysis of SODk0 cells transfected with pBIGF and pcDNABFP in the presence of doxycycline shows little GFP, whereas in the absence of doxycycline, intense GFP staining can be identified. Based on the relative induction of GFP and the efficiency of transfection (Fig. 1), we chose SODk0 as a parental cell line for development of inducible lentiviral vector packaging cell line.

**SODk1 packaging cell line.** To generate an inducible VSV-G pseudotyped lentivirus packaging cell line, we molecularly cloned the VSV-G gene and the entire HIV packaging cassette into tetracycline-inducible vectors. The VSV-G gene was cloned into pBIGF to generate pBIGFVG from which the GFP and the VSV-G are transcribed under the regulation of a bidirectional tetracycline-inducible promoter (Fig. 2). The HIV-1 packaging cassette was cloned into phCMVn to generate pPTK, from which all HIV-1 genes (except the HIV-1 envelope gp120) are expressed under the control of a tetracycline-regulated promoter (Fig. 2). Before establishing a producer cell

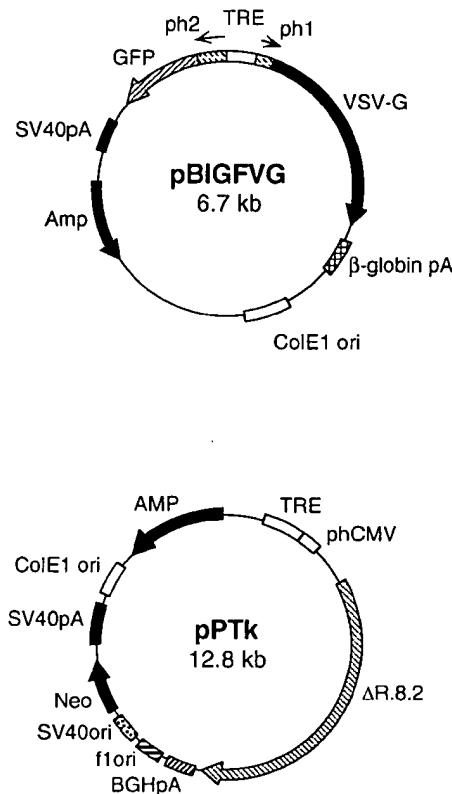


FIG. 2. Inducible envelope and packaging constructs. pBIGFVG is an inducible plasmid that expresses the VSV-G envelope and the GFP genes under the control of a bidirectional tetracycline-inducible promoter. This promoter contains two human CMV minimal promoters (ph1 and ph2) separated by the TRE. Arrows indicate transcription directions. The simian virus 40 (SV40) and  $\beta$ -globin polyadenylation signals are indicated. The pPTK plasmid contains an HIV-1 genomic fragment that encodes all HIV-1 genes excluding the HIV-1 envelope ( $\Delta R.8.2$ ) under the regulation of an inducible promoter. This promoter contains the TRE and the human CMV minimal promoter (pHCMV). Also indicated are the bovine growth hormone polyadenylation signal (BGHPA), the SV40 promoter and origin (SV40ori), the neomycin resistance gene (Neo), the SV40 polyadenylation signal (SV40pA), the ColE1 origin (ColE1 ori), and the f1 origin (f1ori).

line, we wanted to evaluate the potential of inducible packaging systems to produce virus particles. Therefore, we transiently cotransfected SODk0 cells with the inducible packaging plasmid pPTK, the inducible envelope plasmid pBIGFVG, or the previously described pMDG plasmid that expresses the VSV-G envelope under the regulation of the CMV promoter and the vector plasmid pCLCG (12). To standardize vector production, we also generated vector particles by transiently transfecting 293T cells with the vector plasmid pCLCG, pMDG (VSV-G envelope), and the  $\Delta R.8.2$  (packaging) plasmids that were previously described (14). Based on p24 (viral gag) levels in the conditioned media of transfected cells and the actual vector titers (Table 1), we conclude that vector production by the tetracycline-regulated packaging and envelope constructs is similar if not more efficient than the transient three-plasmid transfection method previously employed (10, 12–15).

Encouraged by these results, we embarked on the establishment of a stable packaging cell line. The pPTK packaging construct that also expresses the neomycin resistance gene (Fig. 2) and the pBIGFVG envelope construct were cotransfected into the SODk0 cell line. Thirty-five stably transfected cell clones were isolated and screened for inducible regulation of the VSV-G-GFP and the HIV packaging cassettes. Twenty cell clones in which at least 90% of the cells turned green 48 h

after the withdrawal of doxycycline were selected. The second stage of the screening was aimed at the isolation of the clone in which the highest expression levels of the HIV-1 packaging proteins were obtained following induction. The cells were cultured in the presence or absence of doxycycline, and HIV gag protein production was determined by p24<sup>gag</sup> ELISA. The SODk1 clone was found to be most promising since the p24<sup>gag</sup> concentration in the media was below detection level in the presence of doxycycline and higher than 800 ng/ml without it.

**Induction by sodium butyrate.** Surprisingly, the percentage of SODk1 cells that turned green upon doxycycline withdrawal gradually declined and within 1 month of the isolation of the SODk1 clone, less than 10% of the cells could be induced to express GFP. In parallel, we observed a prolongation in the time required to reach maximal levels of p24<sup>gag</sup>. Interestingly, this phenomenon was common to all 20 cell clones. The fact that the SODk1 cells were resistant to blastocidin and neomycin indicated that the tTA and the packaging plasmids were still integrated in their genome. We attributed the changes in the kinetics and magnitude of SODk1 cells induction to chromatin remodeling. To test this assumption, SODk1 cells were transduced with the pLBFPL vector in which the expression of the BFP is controlled by the HIV-1 LTR. Since the activation of the HIV LTR in 293 cells is tat dependent, we could monitor the induction of the pPTK packaging plasmid by monitoring BFP expression. Expression of pPTK will provide the tat protein, which in turn will activate HIV LTR to transcribe and express BFP. We hypothesized that a potent inhibitor of deacetylation, such as sodium butyrate (22, 24), could revert some of the repressive effects of chromatin on tetracycline-regulated promoters. To test our hypothesis, SODk1Blue cells were cultured in the absence of tetracycline and 48 h later, 5 mM of sodium butyrate was added to half of the samples. By FACS analysis, we evaluated the induction of the pBIGFVG envelope and the pPTK packaging constructs by the expression of the GFP and the BFP, respectively. Figure 3 demonstrates that sodium butyrate has a dramatic effect on the magnitude of induction in SODk1 cells. The time course analysis of SODk1 cells in the presence of sodium butyrate shows that the activation of the pPTK packaging plasmid and the pBIGFVG envelope is relatively synchronized and reaches maximal levels 3 to 4 days after doxycycline withdrawal (Fig. 4). To further evaluate the efficiency of doxycycline regulation of gene expression, we measured the amount of both the Vpr (one of the products of pPTK) and the VSV-G (encoded by pBIGFVG) proteins by Western blot analysis. Constitutive expression of these proteins is not compatible with long-term cell culturing because both

TABLE 1. Vector production by transient three-plasmid transfection of SODk0 cells<sup>a</sup>

Cells	Packaging	Envelope	p24 <sup>gag</sup> level (ng/ml)	Vector titer (IU/ml)
SODk0	pPTK	pMDG	206	$3.5 \times 10^6$
SODk0	pPTK	pBIGFVG	114	$2.0 \times 10^6$
293T	$\Delta R.8.2$	pMDG	53	$1.5 \times 10^6$

<sup>a</sup> To investigate vector production by the inducible envelope and packaging constructs, the vector-producing plasmid pCLCG and the inducible packaging plasmid pPTK were cotransfected to SODk0 cells with either the inducible VSV-G envelope plasmid pBIGFVG or the CMV promoter-regulated VSV-G envelope-producing plasmid pMDG. As a positive control, 293T cells were transiently cotransfected with the vector-producing plasmid pCLCG, the  $\Delta R.8.2$  and the pMDG plasmids that expressed the VSV-G envelope and the HIV-1 packaging genes, respectively, under the control of the CMV promoter. Forty-eight hours posttransfection, vector titers and p24<sup>gag</sup> levels in conditioned media were determined by serial dilutions on 293T cells and by ELISA, respectively.

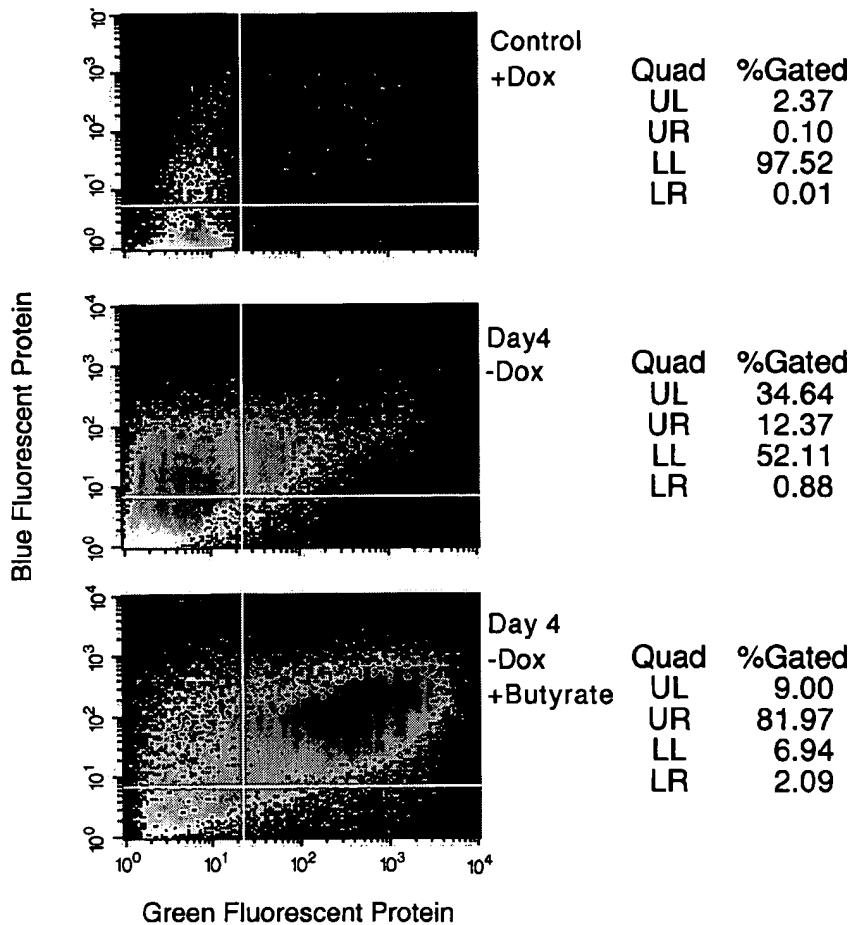


FIG. 3. Induction by sodium butyrate. SODk1Blue cells were induced by doxycycline (DOX) withdrawal in the presence or absence of sodium butyrate. Expression of the BFP and GFP was determined by FACS analysis (y and x axis, respectively) and reflected the activation of the inducible HIV-1 packaging and the VSV-G envelope cassettes, respectively. Noninduced SODk1Blue cells served as a negative control.

proteins are cytostatic or cytotoxic (7, 17, 20). Results shown in Fig. 5A indicate that, although the expression of both proteins is nearly undetectable in the presence of doxycycline (lanes 1 and 3), it is dramatically increased upon doxycycline withdrawal and butyrate treatment (lanes 2 and 4). We therefore conclude that the SODk1 cell line allows regulated production of both HIV viral proteins required for packaging and VSV-G-encoded envelope protein. We next tested if the increased amount of VSV-G protein was in fact due to enhanced transcription of VSV-G gene in the presence of sodium butyrate. Figure 5B (lane 3) shows that, in the absence of doxycycline, the addition of sodium butyrate leads to a greater-than-10-fold increase in VSV-G mRNA. Thus, the addition of sodium butyrate directly affects transcription, which is reflected in the increased virus titers (see below).

**Lentivirus vector producer cell line.** To generate a producer cell line, SODk1 cells were transduced with CLCG lentivirus vector at an MOI of 2. The CLCG vector in the three-plasmid transfections (12) expresses GFP under the control of the CMV promoter; therefore, we could use fluorescence microscopy to determine that between 80 and 90% of SODk1 cells were transduced. Since uninduced SODk1 cells were not producing VSV-G protein, there was presumably no interference to transduction by the CLCG vector containing VSV-G protein. To induce vector production, the cells were cultured in the absence of doxycycline for 24 h followed by the addition of

5 mM of sodium butyrate to half of the samples. Culture medium was replenished every 24 h, and vector production was determined by p24<sup>gag</sup> ELISA and by serial dilutions on 293T cells. Table 2 shows that  $2 \times 10^5$  IU/ml of vector can be generated in 24 h following the addition of sodium butyrate by the stable lentivirus vector producer cell lines. By day 4, nearly  $3 \times 10^6$  IU/ml of recombinant viruses can be obtained; however, vector production at later time points was significantly reduced due to lower cell viability. The producer cells fuse to form syncytia (7). Furthermore, there was a correlation between the actual vector titers that were determined by serial dilutions on 293T cells and the expected titer based on p24<sup>gag</sup> concentration. In the absence of sodium butyrate, the increase in p24<sup>gag</sup> concentration was significantly slower. Although the maximal p24<sup>gag</sup> concentration was not affected by the absence of sodium butyrate, the actual vector titer as determined by serial dilutions on 293T cells was more than 100-fold lower than the titer obtained in the presence of butyrate. Since less than 90% of the SODk1CG1 cells were transduced with the GFP-expressing lentivirus vector, we could increase the titers by reinfecting the cells with the same lentivirus vector (data not shown).

We next examined the lentivirus vector preparations from the SODk1CG1 cell line for the presence of helper virus. As in the past, we have relied on three independent methods: (i) the marker rescue assay; (ii) the *tat* transfer assay on vector-trans-

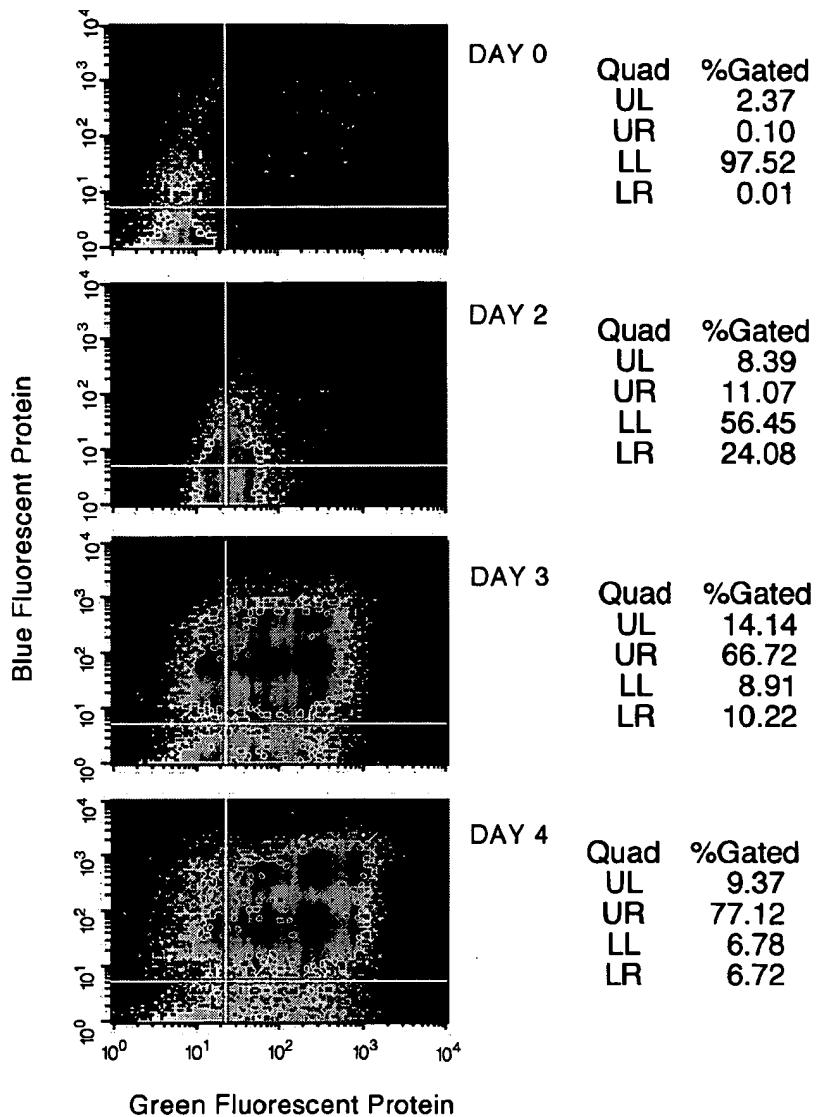


FIG. 4. Induction of SODk1Blue cells. SODk1Blue cells were induced by doxycycline withdrawal in the presence of sodium butyrate. Activation of the VSV-G envelope and the HIV-1 packaging cassettes was reflected by the expression levels of the GFP and the BFP genes, respectively, and was determined at days 0, 2, 3, and 4 postinduction by FACS analysis.

duced P4.2 cells; and (iii) the p24<sup>gag</sup> ELISA on vector-transduced P4.2 cells. No replication-competent helper virus could be detected by these methods (15).

**Properties of viral vectors obtained from the packaging cell line SODk1CG1.** To test the transduction of nondividing cells with lentivirus vectors produced by the SODk1CG1 packaging cell line, human embryonic fibroblasts (HEF) and HeLa cells were growth arrested at the G<sub>1</sub>/S boundary of the cell cycle by culturing in low-serum media (0.1% fetal calf serum) or by aphidicolin treatment, respectively. Table 3 shows that lentivirus vector that was generated by the SODk1CG1 packaging cell line was as efficient at transducing G<sub>1</sub>-arrested cells as proliferating cells.

To investigate if the vector produced by SODk1CG1 packaging cell line can transduce nondividing cells *in vivo*, we concentrated the vector by ultracentrifugation to titers of 10<sup>9</sup> IU/ml. Two microliters of concentrated recombinant vector was injected into the corpus striatum of adult rat brain. Four weeks postinjection, the brains were sectioned and analyzed for GFP

expression by confocal microscopy. Using immunofluorescence staining with antibodies for NeuN and GFAP, we could determine whether the transduced cells were neurons or astrocytes. The results shown in Fig. 6 demonstrate that the vector produced by the SODk1CG1 cell line can efficiently transduce terminally differentiated nondividing neurons. About 60% of the transduced cells were neurons, 32% of the transduced cells were astrocytes, and the remaining 8% showed neither marker. Preferential transduction of neurons over glial cells by lentivirus vectors was previously reported (15), and the mechanism responsible for that has not yet been characterized.

## DISCUSSION

Lentivirus vectors have the ability to deliver and to maintain long-term expression of transgenes in a broad range of tissues *in vivo*. To date, these vectors have been generated by transient three-plasmid transfections (10, 12–15). Although this method allows the generation of high-titer vectors, it limits the amount

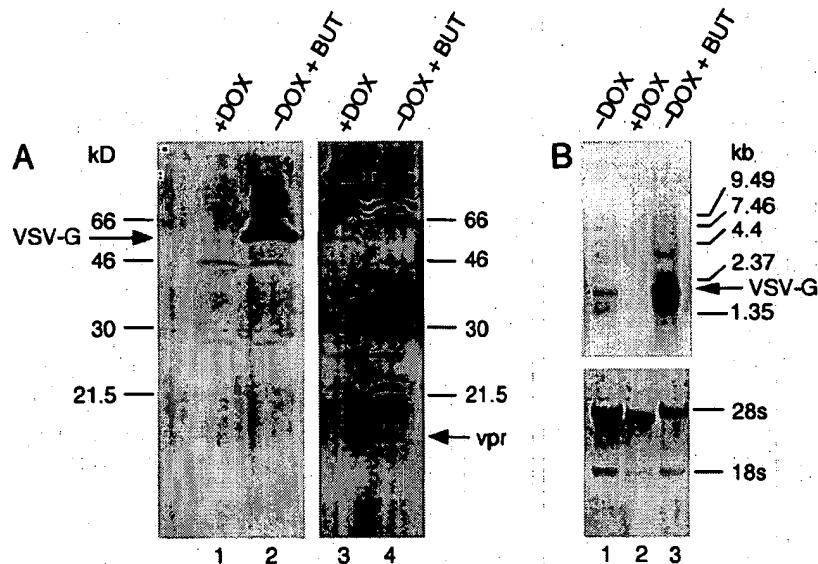


FIG. 5. (A) Western blot analysis of Vpr and VSV-G protein induction. SODk1 cells were induced by doxycycline (DOX) withdrawal and sodium butyrate (BUT) treatment. Three days postinduction, production of the HIV-1 Vpr (lanes 3 and 4) and the VSV-G envelope protein (lanes 1 and 2) in noninduced (lanes 1 and 3) and induced (lanes 2 and 4) cells was determined by Western blot analysis. (B) Northern analysis. Total RNA was extracted from either noninduced SODk1 cells that were cultured in the presence of doxycycline (+DOX) or from SODk1 cells that were induced by doxycycline withdrawal in the presence (-DOX + BUT) or the absence (-DOX) of 5 mM sodium butyrate (upper part of figure). RNA samples were ethidium bromide stained and electrophoresed in a 1.2% agarose gel (lower part of figure). The efficiency of RNA transfer to positively charged membrane was determined by UV fluorescence. The position of the 1.6-kb VSV-G mRNA is indicated with an arrow.

of vector particles that can be produced. It was important, therefore, to develop a stable cell line that can reproducibly generate high-titer lentivirus vectors in amounts that allow lentivirus-based gene therapy on large animal models.

The fact that HIV protease, VSV-G, and HIV *vpr* genes have been reported to be cytotoxic or cytostatic when constitutively expressed mandated the use of an inducible system. Alternatively, we could substitute the VSV-G envelope with a nontoxic envelope gene (e.g., amphotropic *env*) and delete the accessory proteins from the HIV packaging cassette. This could allow us to screen for cell clones resistant to the HIV protease and yet constitutively produce vector particles. Our decision to establish an inducible cell line was based on two major considerations. First, we did not want to lose the major advantages offered by the VSV-G envelope, namely, the broad range of target tissues and the ability for vector concentration by ultracentrifugation (2). Second, we did not want to limit the

use of the lentivirus vector produced by the packaging cell line to tissues that may need complementation for the lack of HIV accessory proteins. Additionally we assumed that the cytotoxic effect of the HIV protease could eventually induce negative selection for cells that express this protein even if the parental cell clone was resistant to the protease. We chose to develop a regulated packaging cell line based on the well-defined and characterized tetracycline-inducible system (8, 9).

In the process of establishing the inducible lentivirus vector packaging cell line, we observed a gradual decline in the reactivation of tetracycline-regulated promoters. The fact that addition of 5 mM sodium butyrate to the culture media overrode the silencing process indicated that this phenomenon was in part the result of changes in chromatin remodeling in the tetracycline-regulated promoters. Interestingly, in the absence of sodium butyrate, p24<sup>gag</sup> production reached its maximal level within 4 to 6 days of doxycycline withdrawal. This result was in accordance with data presented in an earlier report showing that expression of HIV packaging proteins from a tetracycline-regulated promoter reached its maximal levels 6 to 7 days after tetracycline withdrawal (25). Although maximal production of the HIV packaging proteins in the absence of sodium butyrate was not lower than the maximal level obtained in the

TABLE 2. Effect of sodium butyrate on the induction of SODk1CG1 producer cell line<sup>a</sup>

Time of assay (days postinduction)	Result under conditions of -Dox and +sodium butyrate		Result under conditions of -Dox and -sodium butyrate	
	Vector titer (IU/ml)	p24 <sup>gag</sup> level (ng/ml)	Vector titer (IU/ml)	p24 <sup>gag</sup> level (ng/ml)
2	$2 \times 10^5$	120	0	12
3	$1 \times 10^6$	971	$1 \times 10^2$	332
4	$3 \times 10^6$	1,150	$3 \times 10^4$	805
5			$6 \times 10^4$	1,370

<sup>a</sup> SODk1CG1 cells were induced by the withdrawal of doxycycline (-Dox) in the presence (+) or absence (-) of 5 mM sodium butyrate. Media were replenished every 24 h. Conditioned media were assayed daily for vector titers and p24<sup>gag</sup> levels were assayed by serial dilutions on 293T cells and by ELISA, respectively. The titers and p24<sup>gag</sup> levels are averages of results from two independent experiments; there was no more than 25% variation between experiments.

TABLE 3. In vitro transduction of nondividing cells<sup>a</sup>

Cell type	Vector titer (IU/ml) under conditions of:	
	Cycling	G <sub>1</sub> /S arrest
HEF	$5 \times 10^5$	$4 \times 10^5$
HeLa	$1 \times 10^6$	$8 \times 10^5$

<sup>a</sup> Lentivirus vectors generated by the SODk1CG1 producer cell line were titrated by serial dilutions on HEF and HeLa cells arrested in G<sub>1</sub>/S by serum starvation for 48 h or by aphidicolin treatment, respectively. Vector titration on nontransfected HEF and HeLa cells served as a control. The titers are averages of results from two independent experiments; there was no more than 25% variation between the experiments.

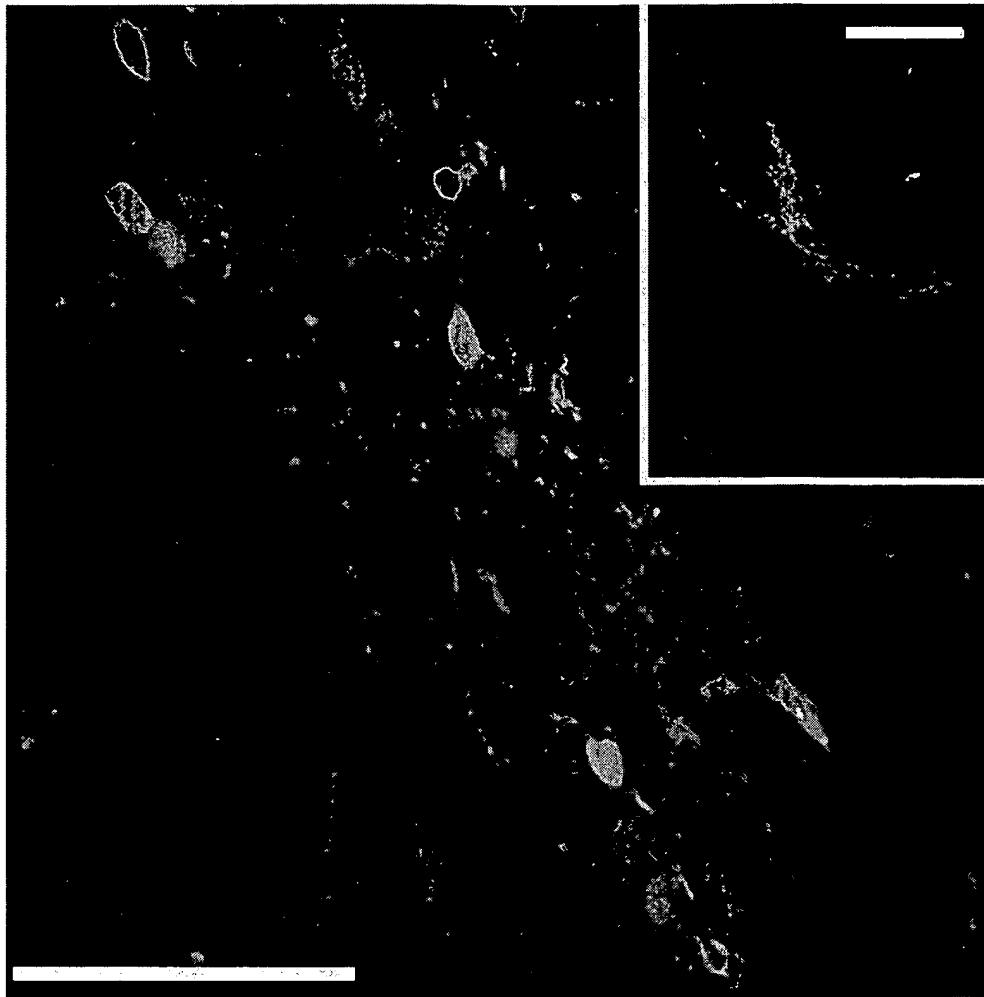


FIG. 6. Transduction of brain of immunocompetent rat. Representative coronal section (40  $\mu$ m thick) of a rat brain with unilateral striatal injection of 2  $\mu$ l of vector expressing GFP 4 weeks after injection. Low magnification (bar = 1 mm) and high magnification (inset) (bar = 100  $\mu$ m) are shown. Double labelling for NeuN (red) and GFAP (blue) was used.

presence of butyrate, vector titers were almost 100-fold lower in the absence of butyrate. This observation can be explained by inefficient induction of the bidirectional tetracycline-regulated promoter from which the GFP and the VSV-G envelope protein are expressed. Clearly, sodium butyrate enhances transcription (Fig. 5B). However, we cannot rule out the possibility that part of the increase in vector titers stems from the effect of butyrate on HIV LTR or other endogenous genes.

Interestingly, secondary transduction of SODk1CG1 cells with the GFP lentivirus vector results in a significant increase in vector titers (9a), which correlated with proportional increase of p24<sup>gag</sup> levels in conditioned media. It is interesting, therefore, to investigate additional rounds of vector transduction and see if p24<sup>gag</sup> levels and vector titers increase. The uncloned populations of SODk1CG1 packaging cell line that have been generated in this study produce high-titer VSV-G pseudotyped lentivirus vectors (up to  $3 \times 10^6$  IU/ml). It is therefore reasonable to assume that even higher titers can be obtained once individual clones can be screened for the highest producer.

In spite of the high-titer vector production, we did not detect helper virus in any of our vector preparations. There are several elements in our cell line that reduce the probability of

generating replication competent virus. (i) The HIV packaging unit, the envelope, and the vector RNA are transcribed from separate expression units. (ii) The VSV-G envelope does not share any sequence homology with the vector or the packaging transcripts. (iii) The transcription of the vector, the envelope, and the packaging unit is limited to the time of induction. (iv) The VSV-G envelope is cytotoxic and therefore reduces the probability of being constitutively expressed. We believe these are important safety features. Further removal of additional viral genes (e.g., *vpr*, *vpu*, *nef*, *vif*, etc.) will add safety features to the lentivirus vector.

In summary, we report here the establishment of a tetracycline-regulated lentivirus vector packaging cell line that reproducibly generates helper-free, high-titer VSV-G pseudotyped vectors. These vectors have a broad spectrum of target tissues and can be concentrated by ultracentrifugation to very high titers (higher than  $10^9$  IU/ml). Vectors produced by the new packaging cell lines efficiently transduced G<sub>1</sub>/S arrested cells *in vitro* and terminally differentiated neurons in brain of immunocompetent rat. The new packaging cell line allows large-scale production of lentivirus vectors and therefore will facilitate human gene therapy efforts.

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## Expression of Human Immunodeficiency Virus Type 1 vif and vpr mRNAs Is Rev-dependent and Regulated by Splicing<sup>1</sup>

STEFAN SCHWARTZ,<sup>\*†</sup> BARBARA K. FELBER,<sup>‡</sup> AND GEORGE N. PAVLAKIS<sup>\*‡</sup>

*\*Human Retrovirus Section, †Human Retrovirus Pathogenesis Group, National Cancer Institute-Frederick Cancer Research and Development Center, ABL-Basic Research Program, Frederick, Maryland 21702-1201, and ‡Department of Virology, Karolinska Institute, Stockholm, Sweden*

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We have analyzed the structure and expression of the HIV-1 vif and vpr mRNAs. The results revealed that the predominant vif and vpr mRNAs belong to the intermediate size class of HIV-1 mRNAs and that their expression is dependent on the presence of Rev protein. In addition, low levels of a small multiply spliced vpr mRNA were produced by HIV-1. cDNA cloning and expression of vpr cDNAs in eucaryotic cells revealed that high levels of Vpr were produced only from the intermediate-size mRNA in the presence of Rev. Thus, as demonstrated for the viral structural proteins, expression of Vif and Vpr is regulated by Rev. The arrangement of the splice sites and the Rev-RRE interaction are responsible for the regulation of viral expression, and especially for the switching from an early stage, producing only or primarily Tat, Rev, and Nef from multiply spliced mRNAs, to a late stage, leading to the production of Gag, Pol, Env, Vpu, Vif, and Vpr from unspliced and partially spliced mRNAs. © 1991 Academic Press, Inc.

### INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) and lentiviruses in general display a complex genomic organization. In contrast to most of the oncoviruses, the lentiviruses contain and express several short open reading frames (ORFs), some of which encode regulatory proteins. To express these different proteins, HIV-1 generates three size classes of RNAs: 9-kb mRNAs coding for Gag and Gag-Pol, 4- to 5-kb mRNAs coding for Env, Vpu, and Tat-1, and 2-kb mRNAs coding for Tat-2, Rev, and Nef (Tat-1 is an alternative functional form of the Tat protein containing 72 amino acids of the first tat exon, produced by intermediate-size mRNAs and found in infected cells (Felber *et al.*, 1990; Schwartz *et al.*, 1990b); Tat-2 is the complete Tat protein of 86 amino acid produced by small multiply spliced mRNAs). All mRNAs are derived from the 9-kb precursor RNA. Three mechanisms are utilized by the virus to express its proteins: ribosomal frameshifting, alternative splicing, and the generation of bicistronic mRNAs. Gag-Pol is expressed from the 9-kb mRNA by ribosomal frameshifting (Varma, 1988). Env and Vpu are expressed from the same intermediate-size

mRNAs by leaky scanning at the vpu AUG (Schwartz *et al.*, 1990b). The regulatory proteins Tat-2, Rev, and Nef are expressed from the first AUG of different small mRNAs generated by alternate splicing (Muesing *et al.*, 1985; Arya *et al.*, 1986; Guatelli *et al.*, 1990; Robert-Guroff *et al.*, 1990; Schwartz *et al.*, 1990a,b). Some of these small mRNAs express more than one protein due to leaky scanning at the AUG of the first ORF (S. Schwartz, B. K. Felber, and G. N. Pavlakis, submitted for publication). In addition, some HIV-1 strains generate other ~2-kb mRNAs by splicing to a small exon in the env region of the virus (Benko *et al.*, 1990; Salfeld *et al.*, 1990; Schwartz *et al.*, 1990a). These mRNAs express alternative forms of the Tat and Rev proteins, named Tev and 6D-Rev (Benko *et al.*, 1990).

All of the above-mentioned mRNAs are subject to positive or negative regulation by the Rev protein. Rev binds to an RNA site (Daly *et al.*, 1989; Zapp *et al.*, 1989; Daefler *et al.*, 1990; Heaphy *et al.*, 1990) within the env region, named the Rev-responsive element (RRE) (Felber *et al.*, 1989; Hadzopoulou-Cladaras *et al.*, 1989). This element was also named CAR (Dayton *et al.*, 1988). Rev promotes the transport and efficient translation of partially spliced HIV mRNAs containing the RRE (Rosen *et al.*, 1988; Emerman *et al.*, 1989; Felber *et al.*, 1989; Hadzopoulou-Cladaras *et al.*, 1989; Hammarskjöld *et al.*, 1989; Malim *et al.*, 1989). In contrast, Rev downregulates the levels of all multiply spliced mRNA species missing RRE (Felber *et al.*, 1990).

Two additional proteins are produced by HIV-1: Vif (Kan *et al.*, 1986; Lee *et al.*, 1986) and Vpr (Wong-Staal

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<sup>2</sup> To whom correspondence and reprint requests should be addressed at National Cancer Institute-Frederick Cancer Research and Development Center, ABL-Basic Research Program, P.O. Box B, Building 539, Room 121, Frederick, MD 21702-1201. Fax: (301) 846-5991.

et al., 1987; Matsuda et al., 1988). The mRNAs expressing these proteins have not been previously identified although different structures of these mRNAs have been proposed (Muessing et al., 1985; Rabson et al., 1985; Arya et al., 1986; Arrigo et al., 1990; Schwartz et al., 1990a). Here we report the identification and regulation of expression of the mRNAs coding for Vif and Vpr. The predominant forms of these mRNAs were of the intermediate size class (4–5 kb) and their expression was Rev-dependent. Cloning and expression of the intermediate vpr cDNA in eukaryotic cells revealed that this mRNA produced high levels of Vpr. In contrast, a minor form of a multiply spliced vpr mRNA produced only low levels of Vpr. The results described here revealed that expression of the vif and vpr mRNAs is Rev-dependent and suggest that both Vif and Vpr are expressed at a late stage in the viral life cycle.

## MATERIALS AND METHODS

### Cells and transfections

Transfections were performed using the HeLa derived cell lines HLtat (Schwartz et al., 1990a), HLFB (Mermer et al., 1990), and HL3T1 (Wright et al., 1986; Felber et al., 1988). HLtat cells constitutively produce the functional Tat-1 protein. HLFB cells contain the rev minus mutant HIV-1 proviral clone, fB (Feinberg et al., 1986; Sodroski et al., 1986a; Hadzopoulou-Cladaras et al., 1989), integrated into their genome. HL3T1 cells contain silent copies of the HIV-1 long terminal repeat (LTR) promoter linked to the chloramphenicol acetyltransferase (CAT) gene. The various plasmids used for transfection were adjusted to 35 µg/ml with pUC19 carrier DNA and were transfected by the calcium phosphate coprecipitation technique (Graham et al., 1973) as described (Felber et al., 1985, 1990).

### cDNA cloning and recombinant plasmids

Total RNA for reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was prepared from HIV-1<sub>HXB2</sub>-infected cells by the guanidine isothiocyanate method (Chirgwin et al., 1979). The RNA was subjected to reverse transcription and PCR amplification (Saiki et al., 1985, 1988) as described (Schwartz et al., 1990a,b) using primers BSS, 710A, and 711A. The exact locations and sequences of the primers are as follows:

BSS	5'-GGCTTGCTGAAGCGCGCACGGCAAGAGG-3' (246–273)
710A	5'-CTGCTAGTGCCAAGTATTG-3' (5040–5022)

### 711A 5'-CTATTATCTTGTATTACTAC-3' (4539–4520)

The numbering of the HIV-1 genome (clone HXB2) starts at the first nucleotide (nt) of the R-region of the 5' LTR in the corrected HXB2R sequence (Ratner et al., 1985, 1987; Myers, 1990; Solomin et al., 1990). The amplification products were digested with *Bss*HII and cloned as *Bss*HII-to-blunt end fragments in a modified Bluescript KS<sup>+</sup> vector (Stratagene). Clones were screened by colony hybridization and restriction enzyme analysis. Double-stranded DNA sequencing of miniplasmid DNA preparations from appropriate clones was performed using the Sequenase kit (United States Biochemicals) as described (Schwartz et al., 1990a,b).

pNL1.3A.7(vpr) was constructed by subcloning the *Stu*I–*Sa*I fragment of pCV12 (Arya et al., 1986) into the *Stu*- and *Sa*I-digested tat cDNA pNL1.3.4.7 (Schwartz et al., 1990a). pNL1.3AE(vpr) was generated by replacing the sequences between the unique *Sa*I and *Bam*HI restriction sites in pNL1.3A.7(vpr) with the genomic *Sa*II–*Bam*HI fragment. These plasmids expressed mRNAs that were identical to those produced by the intact provirus. pNL1.3AEfB(vpr) was generated by digestion of pNL1.3AE(vpr) with *Bam*HI, filling in of the 5' overhang, and religation. The following plasmids have been described previously: pNL1.5E(vpu/env) (Schwartz et al., 1990b), pNL1.4.7(tat) (Schwartz et al., 1990a), pNL1.4A.7(rev) (Schwartz et al., 1990a), pNL1.5.7(nef) (Schwartz et al., 1990a), HXB2 (Ratner et al., 1985, 1987), JY1 (Yourno et al., 1989), pNL43 (Adachi et al., 1986), and fB (Feinberg et al., 1986; Sodroski et al., 1986a; Hadzopoulou-Cladaras et al., 1989).

### RNA extractions and Northern blots

Total RNA for Northern blot analysis was extracted one day post transfection by the heparin/DNase procedure (Krawczyk et al., 1987). The blots were hybridized to nick-translated PCR amplified DNA fragments (Saiki et al., 1985, 1988). PCR was carried out for 20 cycles under standard conditions with 200 ng of pHXB2 as DNA template. The resulting products were gel-purified on acrylamide gels. The probe hybridizing to the 3' end of all viral mRNAs spans nt 8304–9008. The vif/vpr probe spans nt 5095–5315, and the vif probe spans nt 4520–4904. The amount of radioactivity on the blots was quantitated using the AMBIS radioapalytic imaging system.

### Immunoprecipitations, p24<sup>gag</sup> antigen assays, and CAT assays

One day after transfection, HLtat cells (Schwartz et al., 1990a) were metabolically labeled with [<sup>35</sup>S]me-

thionine for 3 hr. The cells were lysed in 0.5× RIPA buffer and analyzed by immunoprecipitations on 12.5% denaturing polyacrylamide gels as described (Felber *et al.*, 1990). For p24<sup>gag</sup> quantitations, cells were lysed in 100 mM Tris-HCl, pH 7.4, and 0.1% Triton X-100 and cleared by centrifugation (Solomin *et al.*, 1990). The p24<sup>gag</sup> antigen capture assay (Dupont, NEN) was performed according to the manufacturer's recommendations. All Rev-expressing plasmids were serially diluted and transfected into HLFb cells (Mermmer *et al.*, 1990) to determine the amount of plasmid that resulted in p24<sup>gag</sup> response in the linear range. p24<sup>gag</sup> values were then normalized to the amount of plasmid used for transfection. CAT assays were performed as described (Gorman *et al.*, 1982) and the range of linear response of CAT to Tat protein was determined from serial dilutions of the tat cDNA pNL1.4.7 (Schwartz *et al.*, 1990a) as described (S. Schwartz, B. K. Felber, and G. N. Pavlakis, submitted for publication).

## RESULTS

### The vif and the vpr mRNAs belong to the intermediate size class of HIV-1 mRNAs

As shown in Fig. 1, the structure of the HIV-1 genome is complex and contains several ORFs in addition to gag, pol, and env. Although most of the mRNAs produced by HIV-1 have been characterized (Muesing *et al.*, 1990a,b), the mRNAs expressing Vif and Vpr proteins have not been identified unambiguously and the regulation of expression of these mRNAs is not known. To investigate how Vif and Vpr are expressed by HIV-1, we analyzed the expression of vif and vpr mRNAs in HeLa cells transfected with three different infectious HIV-1 molecular clones, HXB2 (Ratner *et al.*, 1985, 1987), JY1 (Youno *et al.*, 1989), and NL43 (Adachi *et al.*, 1986). RNA was prepared from the transfected cells and subjected to Northern blot analysis. The blots were hybridized either to a vif/vpr probe, which is complementary to the region between exons 3 and 4 and contains both vif and vpr coding sequences, or to a probe that hybridizes between exons 2 and 3 and contains only vif coding sequences (Fig. 2). Figure 3A shows that in addition to the 9-kb unspliced HIV-1 mRNA, two intermediate-size mRNAs were detected by the vif/vpr probe. Other investigators have also detected additional intermediate sized mRNAs by Northern blot analysis (Rabson *et al.*, 1985). The lower band was predicted to be the vpr mRNA and the upper band was predicted to be the vif mRNA. To confirm that the upper band represented the vif mRNA, the blots were rehybridized with the vif probe. The results showed that this probe hybridized only to the upper band, verifying that this was the vif mRNA (Fig. 3B).

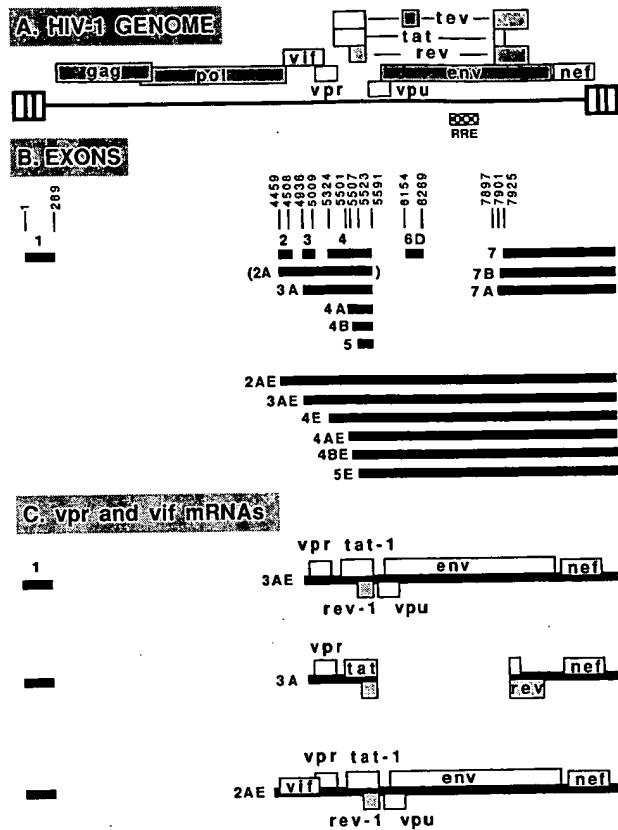


FIG. 1. (A) Genomic organization of HIV-1. Open or shaded boxes represent ORFs. (B) Black bars indicate exons present in HIV-1 mRNAs. They are named according to Muesing *et al.* (1985) and Schwartz *et al.* (1990a,b). Nucleotide positions of splice donors and acceptors are indicated. Numbering starts at the first nucleotide of the R region of the 5' LTR. Exon 2A (in parentheses) (Arya *et al.*, 1986) has not been detected in any mRNA by Northern blot analysis. (C) Structures of the two vpr mRNAs 1.3AE and 1.3A.7 and the vif mRNA 1.2AE. These mRNAs are named for the exons they contain. Open boxes indicate ORFs.

Both the vpr and the vif mRNAs were produced by all three viral clones HXB2, NL43, and JY1 (Figs. 3A and 3B). The predicted structures of these mRNAs are shown in Fig. 1C. The lanes shown in Figs. 3A and 3B are overexposed to detect minor RNA species.

In addition to the RNAs described above, the vif/vpr probe hybridized to a lower band migrating with the class of multiply spliced mRNAs (Fig. 3A). This mRNA was not detected by the vif-specific probe (Fig. 3B). This indicated the presence of low levels of a vpr-encoding mRNA missing the env region. The structure of this mRNA is shown in Fig. 1C {1.3A.7(vpr)}. The predicted size of 1.3A.7(vpr) is in agreement with the migration of the lower band in Fig. 3A. Vif mRNAs belonging to the multiply spliced class of mRNAs could not be detected by Northern analysis (Fig. 3B). Previous cloning results have also suggested the presence of multi-

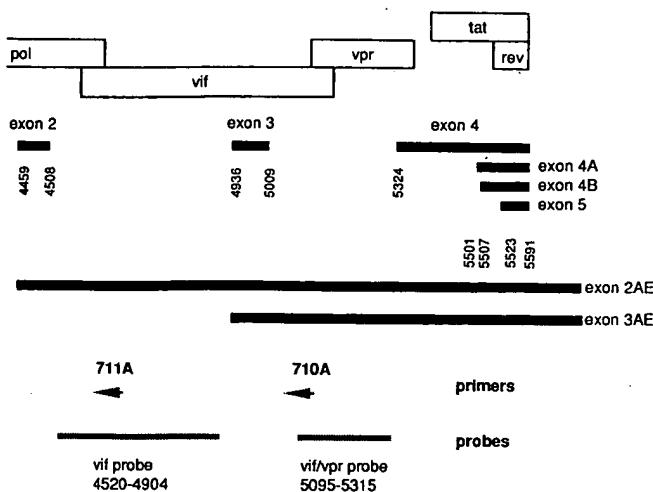


FIG. 2. Organization of the central portion of the HIV-1 genome. Open boxes indicate ORFs and black bars indicate exons. Nucleotide positions of the splice sites are indicated. Locations of the oligonucleotide primers 710A and 711A are indicated by arrows, and the vif and vif/vpr probes are indicated as gray bars.

ply spliced vif and vpr mRNA species (Arya *et al.*, 1986; Schwartz *et al.*, 1990a). The results described here revealed that vif- and vpr-containing mRNAs were found primarily among the intermediate-size HIV-1 mRNAs.

#### Expression of vif and vpr mRNAs is Rev-dependent

To investigate whether the expression of vif and vpr mRNAs was dependent on Rev, we analyzed RNA from HeLa cells transfected with the rev minus mutant proviral clone fB (Feinberg *et al.*, 1986; Sodroski *et al.*, 1986a; Hadzopoulou-Cladaras *et al.*, 1989) (Figs. 3A and 3B). The results revealed that only very low levels

of the vif and the vpr mRNAs were found in cells transfected with fB, while high levels of these mRNAs were found in cells transfected with the wild-type viral clones (Figs. 3A and 3B). Hybridization of the blots to a 3' end probe that detects all mRNAs verified that fB expressed high levels of the small multiply spliced mRNAs and low levels of the partially spliced and unspliced mRNAs (Hadzopoulou-Cladaras *et al.*, 1989) (data not shown). In addition, we analyzed RNA produced by a HeLa-derived cell line constitutively expressing the rev minus fB provirus (HLfB cells; Mermer *et al.*, 1990). Vif and vpr mRNAs were present only in very low levels in HLfB cells (Fig. 3C). Complementation in trans by Rev resulted in higher levels of these mRNAs (Fig. 3C). These results further demonstrated that expression of vif and vpr mRNAs was Rev-dependent.

#### Identification of splice sites of the vif and vpr mRNAs

As shown in Figs. 1 and 2, the vpr ORF is directly preceded by the small noncoding exon 3, which is present in some of the mRNAs coding for Tat, Rev, and Nef (Muesing *et al.*, 1985; Arya *et al.*, 1986; Guatelli *et al.*, 1990; Robert-Guroff *et al.*, 1990; Schwartz *et al.*, 1990a,b). We next investigated if the splice acceptor of exon 3 (nt 4936) was utilized to generate the vpr mRNAs as has been suggested previously (Arrigo *et al.*, 1990; Schwartz *et al.*, 1990a). RNA from HIV-1-infected cells was analyzed for the presence of mRNAs that splice from exon 1 to the splice acceptor of exon 3 without further splicing. Such mRNAs would have the vpr ORF as the first intact ORF. Partial cDNAs were synthesized from total RNA of HIV-1-infected cells (clone HXB2; Ratner *et al.*, 1985, 1987), and subjected

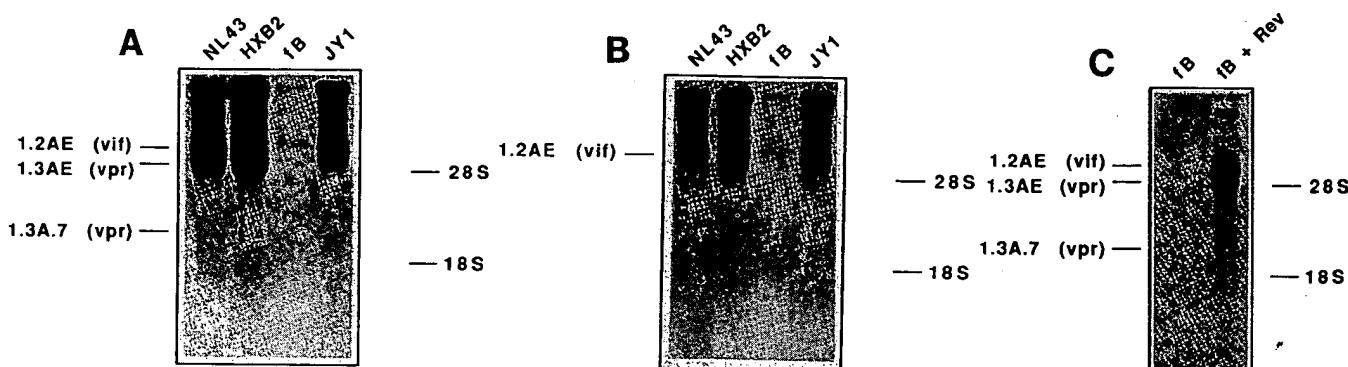


FIG. 3. (A) Northern blot analysis of RNA from HLtat cells transfected with the different molecular clones of HIV-1 indicated at the top. The blot was hybridized to the vif/vpr probe, which contains nt 5095-5315 (see Fig. 2) and detects both vif and vpr mRNAs. (B) Hybridization of the same RNA samples as in (A) to a vif specific probe containing nt 4520-4904 (Fig. 2). (C) Northern blot analysis of RNA from mock-transfected HLfB cells or HLfB cells transfected with the rev expression plasmid pNL1.4A.7. The blot was hybridized to the vif/vpr probe, which detects both vif and vpr mRNAs. Locations of the 28S and 18S rRNAs are indicated to the right, and locations of the vpr and vif mRNAs are indicated to the left. The smaller vpr RNA is barely detectable in this exposure.

to PCR amplification using the primer pair BSS-710A (Fig. 2). Cloning and sequencing of the partial cDNAs confirmed the presence of mRNAs utilizing the splice acceptor, but not the splice donor, of exon 3. Such mRNAs could express Vpr protein. We did not find any alternative splice acceptors immediately upstream of the vpr ORF and therefore concluded that the splice acceptor of exon 3 is utilized to generate the vpr mRNAs.

The predicted structure of the intermediate-size vpr mRNA is shown in Fig. 1C. The exon that starts at the splice acceptor of exon 3 and continues to the poly(A) site at the 3' end of the genome was named 3AE. The mRNA containing this exon was named 1.3AE(vpr) after the exons it contained. Its predicted size of 4.4 kb was consistent with the size of the vpr mRNA detected by Northern blot analysis (Fig. 3A). Because the common splice donor at nt 5591, downstream of the vpr ORF, could splice to exon 7 (nt 7925), which is the last exon of the genome (Fig. 1), a second vpr mRNA was predicted. This mRNA would belong to the class of the small multiply spliced HIV-1 mRNAs and would not contain the RRE (Dayton *et al.*, 1988; Rosen *et al.*, 1988; Emerman *et al.*, 1989; Felber *et al.*, 1989; Hadzopoulou-Cladaras *et al.*, 1989; Hammarström *et al.*, 1989; Malim *et al.*, 1989). The exon that starts at the splice acceptor of exon 3 and continues unspliced up to the splice donor at nt 5591 was named 3A, and the mRNA containing this exon was named 1.3A.7(vpr) (Fig. 1). This mRNA has a predicted size of 2.1 kb, which is in agreement with the size of the small RNA detected by the vpr probe (Fig. 3A). Both mRNAs 1.3A.7 and 1.3AE contain the splice donor of exon 3 and the splice acceptors of exons 4, 4A, 4B, and 5 and could therefore be further spliced (Figs. 1 and 2). Only mRNA 1.3AE contain the RRE and could therefore be regulated by the viral Rev transactivator. mRNA 1.3A.7 cannot be affected by Rev and remains vulnerable to further splicing. This would explain the low levels of mRNA 1.3A.7 produced by the virus (Fig. 3A).

The location of the vif ORF downstream of the non-coding exon 2 suggested a similar structure of the vif mRNA (Arrigo *et al.*, 1990; Schwartz *et al.*, 1990a). This predicted that the splice acceptor of exon 2 is utilized to generate an exon named 2AE (Fig. 1), which would be present in the intermediate-size 4.4-kb vif mRNA detected in transfected cells (Fig. 3B). PCR analysis of HIV-1 mRNAs using the primer pair BSS-711A (Fig. 2) as well as other primers, revealed that the exon 2 splice acceptor was the only recognized splice site in the vicinity of the vif ORF (data not shown). These results, in combination with the observed size of the mRNA hybridizing to the specific vif probe (Fig. 3B), revealed the structure of the vif mRNA (Fig. 1C). We

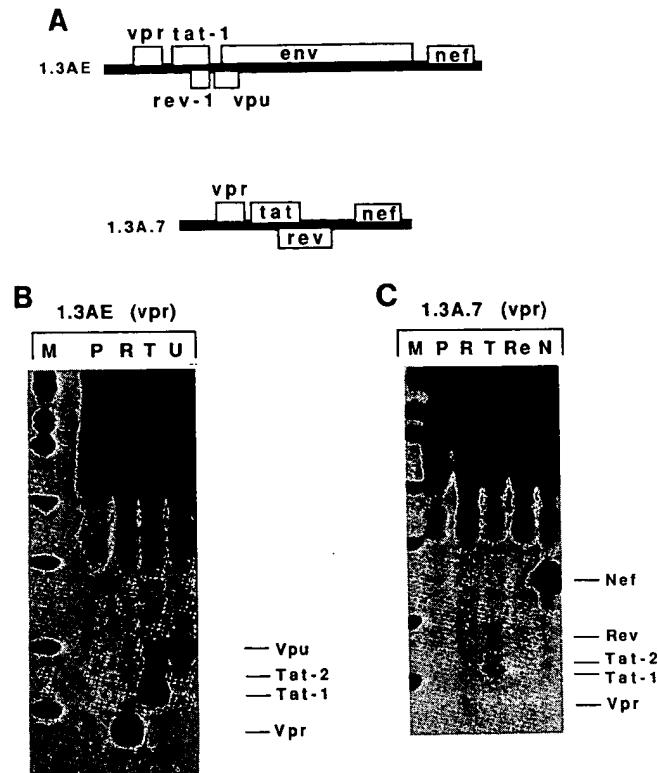


Fig. 4. Expression of cDNA pNL1.3AE(vpr) or pNL1.3A.7(vpr) in HLtat cells. (A) The structures of mRNAs 1.3AE and 1.3A.7 are shown. (B) Immunoprecipitation of Vpr, Tat, and Vpu from HLtat cells transfected with pNL1.3AE. (C) Immunoprecipitations of Vpr, Tat, Rev, and Nef from cells transfected with pNL1.3A.7. Tat-1 is the truncated one-exon Tat protein produced by the HLtat cells and from pNL1.3AE; Tat-2 is the two-exon Tat protein produced from the multiply spliced tat mRNA. M, molecular weight markers; P, preimmune serum; R, anti-Vpr antiserum; T, anti-Tat antiserum; U, anti-Vpu antiserum; Re, anti-Rev antiserum; N, anti-Nef antiserum.

concluded that the vpr mRNAs utilize the splice acceptor of exon 3 and that the vif mRNAs utilize the splice acceptor of exon 2.

#### Expression of vpr cDNAs in eukaryotic cells

To further analyze regulation of Vpr expression, we investigated whether both the small vpr mRNA 1.3A.7 and the intermediate-size vpr mRNA 1.3AE were able to produce Vpr. Eukaryotic expression plasmids producing these mRNAs were generated by replacing the missing sequences 3' of primer 710A and 5' of primer BSS in the partial cDNAs. This resulted in plasmids pNL1.3A.7 and pNL1.3AE (Fig. 1). Expression of 1.3AE in HLtat cells (Schwartz *et al.*, 1990a) revealed that high levels of Vpr were expressed from this mRNA (Fig. 4B). In contrast, pNL1.3A.7 produced very low levels of Vpr compared to mRNA 1.3AE (Fig. 4C). This indicated that the major Vpr-producing HIV-1 mRNA was 1.3AE.

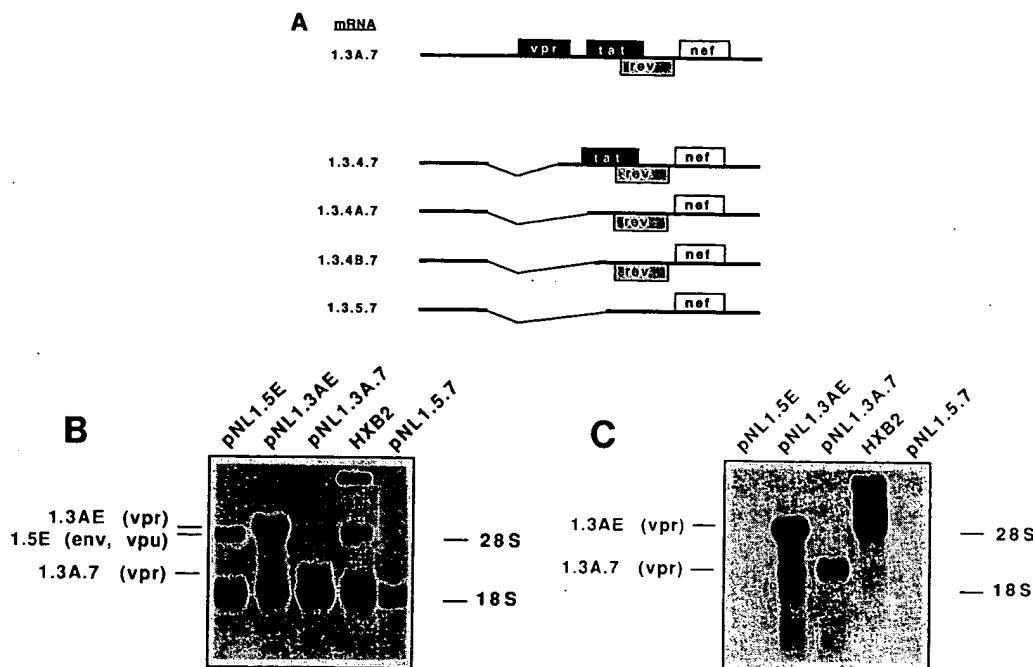


FIG. 5. (A) Structure and coding potential of the mRNAs generated by pNL1.3A.7(vpr). The mRNAs are named to indicate the exons they contain. The ORFs present on the mRNAs are indicated by boxes. (B) Northern blot analysis of RNA from HLtat cells transfected with the various cDNAs shown at the top. Hybridization to a probe complementary to the 3' end of all the mRNAs (nt 8304–9008) detects all mRNAs produced by HIV-1. The positions of some of the RNAs produced by the cDNAs are shown to the left, and the positions of the 28 S and 18 S rRNAs are shown to the right. (C) Hybridization of the same RNA samples as in (B) to the vif/vpr probe (see Fig. 2).

The inability of pNL1.3A.7 to express Vpr efficiently suggested that this mRNA underwent further splicing, which deleted the vpr ORF. In contrast to mRNA 1.3AE, mRNA 1.3A.7 does not contain the RRE and therefore is not subject to regulation by Rev. The predicted splice products of mRNA 1.3A.7 are shown in Fig. 5A. It has previously been shown that mRNA 1.3.4.7 produces Tat, mRNAs 1.3.4A.7 and 1.3.4B.7 produce Rev and Nef, and mRNA 1.3.5.7 produces only Nef (Schwartz *et al.*, 1990a). To verify that 1.3A.7 splices, we analyzed the RNAs expressed from pNL1.3A.7, pNL1.3AE, and other cDNA constructs by Northern blot hybridization, using either a probe that detects the 3' end of all differently spliced HIV-1 mRNAs or the vif/vpr probe described in Fig. 2. Figure 5B shows that pNL1.3A.7 produced two RNAs detected by the probe hybridizing to the 3' end of the mRNAs. The upper band corresponded in size to the unspliced 1.3A.7 mRNA, and the lower band corresponded to the tat, rev, and nef mRNAs. Hybridization of the blot to the vif/vpr specific probe revealed that only the upper band was detected by this probe (Fig. 5C). The vif/vpr probe did not hybridize to the env mRNA 1.5E (Schwartz *et al.*, 1990b), nor did it hybridize to the multiply spliced Nef mRNA 1.5.7 (Schwartz *et al.*, 1990a), demonstrating that this probe is specific for vpr-containing mRNAs (Fig. 5C). These results verified

the identity of the small band as vpr mRNA 1.3A.7. Quantitation of Northern blots revealed that one third of the total RNA produced from pNL1.3A.7 remained unspliced. In addition, RT-PCR analysis after expression of 1.3A.7 confirmed the occurrence of further splicing, especially to the nef splice acceptor at position 5523 (data not shown). These results showed that mRNA 1.3A.7 is not terminally spliced and is rapidly spliced into the smaller tat, rev, and nef mRNAs.

Analysis of RNA expression from pNL1.3AE revealed high levels of an intermediate-size mRNA detected both by the 3' end probe (Fig. 5B) and by the vif/vpr-specific probe (Fig. 5C). This band comigrated with the intermediate-size vpr mRNAs detected in the cells transfected with the proviral clones (Fig. 3A). pNL1.3AE also produced the small multiply spliced mRNAs identified as tat, rev, and nef mRNAs, since they hybridized to the 3'-end probe but not to the vpr probe. Interestingly, only a small fraction of the spliced mRNAs generated by pNL1.3AE migrated as mRNA 1.3A.7 (Fig. 5C), further demonstrating that 1.3A.7 is rapidly spliced to produce tat, rev, and nef mRNAs.

We next investigated whether Vpr expression from mRNA 1.3AE was Rev-dependent. Two Rev-expressing mRNAs, 1.3.4A.7 and 1.3.4B.7, could be generated by splicing of 1.3AE. Inactivation of the rev ORF by a frameshift mutation in pNL1.3AE should therefore

result in the loss of Vpr expression from this plasmid. The resulting plasmid, pNL1.3AEfB, expressed only very low levels of Vpr (data not shown) as expected, verifying that expression of Vpr from mRNA 1.3AE requires the presence of Rev protein *in trans* and the RRE *in cis*. We concluded that Vpr is expressed primarily from the intermediate-size mRNA and that Vpr expression is regulated by Rev.

#### Vpr mRNAs are further spliced to tat, rev, and nef mRNAs in the absence of Rev

To verify that the mRNAs expressing tat, rev, and nef were generated from the vpr mRNA 1.3A.7, HLtat cells were transfected with pNL1.3A.7 and metabolically labeled with [<sup>35</sup>S]methionine. Expression of Tat, Rev, and Nef was determined by immunoprecipitations. As shown in Fig. 4, low levels of Tat and high levels of Nef were detected, indicating that nef mRNA 1.3.5.7 represented the major splice product. Rev could not be detected by immunoprecipitation (see below).

Interestingly, immunoprecipitation of Tat-1, Vpu (Fig. 4), and Env (data not shown) from cells transfected with pNL1.3AE(vpr) revealed that high levels of these proteins were produced, indicating that this mRNA was also further spliced to generate other intermediate-size mRNAs. These results suggested that splicing from the splice donor of exon 3 (nt 5009) preceded splicing from the splice donor at nt 5591.

To examine the possibility that pNL1.3A.7(vpr) might produce low levels of Rev protein that were below the sensitivity of the immunoprecipitation technique, a more sensitive Rev-dependent complementation assay was used to measure low levels of Rev production (Mermer *et al.*, 1990) (S. Schwartz, B. K. Felber, and G. N. Pavlakis, submitted for publication). A HeLa-derived cell line (HLfB) containing stably integrated copies of the rev minus mutant HIV-1 provirus fB was transfected with pNL1.3A.7(vpr) or the rev cDNA pNL1.4A.7 (Schwartz *et al.*, 1990a) and p24<sup>gag</sup> production was quantitated by the p24<sup>gag</sup> antigen capture assay. As discussed in detail elsewhere, complementation *in trans* by Rev results in production of p24<sup>gag</sup> by this cell line in a dose-dependent manner (S. Schwartz, B. K. Felber, and G. N. Pavlakis, submitted for publication). Table 1 shows that pNL1.3A.7(vpr) produced about 2% of the amount of Rev expressed by the rev cDNA 1.4A.7, thus confirming the production of Rev protein.

To estimate the efficiency of splicing of exon 3 to exon 4, which generates the tat mRNA 1.3.4.7 (Schwartz *et al.*, 1990a), we quantitated Tat production from pNL1.3A.7(vpr) using a sensitive functional assay for Tat. pNL1.3A.7 was transfected into HL3T1 cells (Wright *et al.*, 1986; Felber *et al.*, 1988), a HeLa derived

TABLE 1

LEVELS OF Tat AND Rev PROTEINS PRODUCED BY DIFFERENT PLASMIDS MEASURED BY FUNCTIONAL ASSAYS (CAT AND p24<sup>gag</sup>, RESPECTIVELY)

Plasmid	p24 <sup>gag</sup> <sup>a</sup>	CAT <sup>b</sup>
pNL1.3A.7 (vpr)	2%	10%
pNL1.4A.7 (rev)	100%	0%
pNL1.4.7 (tat)	0.2%	100%
pUC19	0%	0%

<sup>a</sup> p24<sup>gag</sup> was measured after transfection of HLfB cells, which contain the rev minus mutant HIV-1 proviral clone (fB) stably integrated into the genome. p24<sup>gag</sup> response is expressed as a percentage of the response obtained after transfection with the rev cDNA pNL1.4A.7 (Schwartz *et al.*, 1990a).

<sup>b</sup> CAT activity was measured after transfection of HL3T1 cells, which contain silent copies of the HIV-1 LTR promoter linked to the CAT gene. The CAT value is expressed as a percentage of the response obtained after transfection with the tat cDNA pNL1.4.7 (Schwartz *et al.*, 1990a).

cell line containing silent copies of the HIV-1 LTR promoter linked to the CAT gene. As a result of Tat expression, CAT is produced in a linear, dose-dependent manner (S. Schwartz, B. K. Felber, and G. N. Pavlakis, submitted for publication). Tat production from pNL1.3A.7(vpr) was determined and compared to Tat expression from the tat cDNA pNL1.4.7 (Schwartz *et al.*, 1990a). The results revealed that pNL1.3A.7 produced 10% of the amount of Tat expressed by the tat cDNA (Table 1). These results demonstrated that splicing from the splice donor of exon 3 occurred, which generated tat, rev, and nef mRNAs. Splicing occurred primarily to exon 5 and to a lesser extent to exons 4, 4A, and 4B.

#### DISCUSSION

In this work we have shown that the splice acceptor of exon 2 at position 4459 is used for the generation of vif mRNA. Similarly, the splice acceptor of exon 3 at position 4936 is used for the generation of vpr mRNA. No other splice sites were detected by RT-PCR analysis. HIV-1 expresses at least six regulatory and accessory proteins. Upstream of each ORF encoding vif, vpr, tat, rev, vpu, and nef is a splice acceptor that generates at least one mRNA for each ORF. In contrast to the tat, rev, and nef ORFs, the vif and vpr ORFs are preceded by splice donors of exons 2 and 3, respectively. Therefore, further splicing involving the splice donor of exon 2 or exon 3 joins these small, noncoding exons to downstream exons such as exons 4, 4A, 4B, and 5, resulting in tat, rev, and nef or vpu/env mRNAs. This explains the presence of three variants of the tat, rev, nef, and vpu/env mRNAs, (containing exon 2 or exon 3

or neither of these exons) found in infected cells (Muesing *et al.*, 1985; Arya *et al.*, 1986; Guatelli *et al.*, 1990; Robert-Guroff *et al.*, 1990; Schwartz *et al.*, 1990a,b). As a result, mRNAs that are spliced to exon 2 or 3 may or may not generate vif or vpr mRNAs, depending on the presence of Rev protein. This arrangement of the HIV-1 genome allows the presence of additional functional splice sites and ORFs without inhibiting expression of downstream ORFs. The data described here established that the splice donors of exons 2 and 3 have an important regulatory function, since their utilization leads to further splicing of vif and vpr mRNAs and the generation of tat, rev, and nef mRNAs in the absence of Rev protein.

The vpr mRNA is of the intermediate size class and its expression depends on Rev protein (see Fig. 3), therefore expression of Vif is regulated similarly to that of Vpr. In contrast to the vpr mRNA 1.3A.7, the predicted multiply spliced vif mRNA 1.2A.7 could not be detected by Northern blot analysis, indicating that it is efficiently spliced. This apparent difference in splicing efficiency may be explained by the presence of additional splice sites in the vif mRNA. It may therefore be present in low levels which are undetectable by Northern blot analysis. Detection of low levels of the multiply spliced vpr mRNA 1.3A.7 is most likely due to the fact that retroviral splice sites are less efficient than their cellular counterparts. As a result, a measurable fraction of the 1.3A.7 mRNA remains unspliced. It has been shown that efficiently spliced cellular mRNAs, such as the  $\beta$ -globin mRNA, are not regulated by HIV-1 Rev and RRE, suggesting that "slow" splicing is a prerequisite for Rev regulation (Chang *et al.*, 1989). Although most of the 1.3A.7 transcript is further spliced, the remaining unspliced mRNA produces small but detectable amounts of Vpr protein. Therefore, this mRNA may not contain the same set of inhibitory sequences found in gag and env mRNAs. Further detailed analysis of inhibitory sequences within the gag mRNA supports this interpretation (S. Schwartz, B. K. Felber, and G. N. Pavlakis, submitted for publication).

Our data show that the mRNAs expressing Vif and Vpr belong primarily to the intermediate size class of HIV-1 mRNAs. Expression of these mRNAs was Rev-dependent, indicating that vif and vpr are expressed late in the viral life cycle, together with gag, pol, env, and vpu. This is consistent with the function of the Vif protein, which appears to be required for full infectivity of the viral particles (Sodroski *et al.*, 1986b; Fisher *et al.*, 1987). Vpr is associated with viral particles (Cohen *et al.*, 1990a; Yuan *et al.*, 1990) and may be a structural component of the virion. Vpr minus mutant molecular clones of HIV-1 replicate more slowly than the wild-type virus (Dedera *et al.*, 1989; Ogawa *et al.*, 1989;

Cohen *et al.*, 1990b), and it has been suggested that Vpr may be a regulatory protein, since it activates expression from various viral promoters (two- to threefold (Cohen *et al.*, 1990b)). Since Vpr is present in the virion, it has been argued that this protein may act early after infection.

Interestingly, other lentiviruses such as simian immunodeficiency virus (SIV), caprine arthritis-encephalitis virus (CAEV), feline immunodeficiency virus (FIV), and visna produce mRNAs containing small noncoding exons within the pol region of the virus. These mRNAs are similar in structure to the tat, rev, and nef mRNAs of HIV-1, containing the noncoding exons 2 or 3. The SIV genome contains two noncoding exons upstream of vif and vpx (Colombini *et al.*, 1989; Viglianti *et al.*, 1990). Visna (Mazarin *et al.*, 1988; Davis *et al.*, 1989), CAEV (Saltarelli *et al.*, 1990), and FIV (Phillips *et al.*, 1990) genomes also contain a small noncoding exon positioned upstream of an ORF, which has been named Q in the case of visna virus (Sonigo *et al.*, 1985). This suggests that expression of the Q ORF may be subjected to a similar regulation as the HIV-1 vif and vpr ORFs. The more distantly related spuma retroviruses also express multiply spliced mRNAs, some of which appear to contain small noncoding exons (Muranyi *et al.*, 1991). It remains to be investigated whether expression of this virus is regulated by similar mechanisms as HIV-1 and many other lentiviruses.

The results presented in this report complete the identification, characterization, and study of regulation of mRNAs producing all HIV-1 proteins (Benko *et al.*, 1990; Felber *et al.*, 1990; Schwartz *et al.*, 1990a,b); (S. Schwartz, B. K. Felber, and G. N. Pavlakis, submitted for publication). These data are valuable for understanding the regulation of HIV-1 expression and for studying the expression of individual viral proteins in infected cells. These findings further demonstrate the complexity of the regulation of lentiviral gene expression. Since HIV-1 is the prototype lentivirus, these results may help the elucidation of mechanisms of lentiviral expression and regulation.

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also presented evidence supporting that vif and vpr expression is Rev-dependent.

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## Involvement of Human Immunodeficiency Virus Type-1 Splice Sites in the Cytoplasmic Accumulation of Viral RNA

Keith T. Borg, Justin P. Favaro, and Salvatore J. Arrigo<sup>1</sup>

Department of Microbiology and Immunology, Medical University of South Carolina, Charleston, South Carolina 29425-2230

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To define the role of human immunodeficiency virus type 1 splice sites in the cytoplasmic accumulation of viral RNAs, sequential deletion mutagenesis on an infectious proviral clone of HIV-1 was performed. Deletion of the majority of intron sequences, containing previously identified CRS, did not attenuate CRS activity. Retention of either the first or second *tat* intron preserved CRS activity. RNAs containing splice donor sequences, in the absence of known downstream splice acceptor sequences, retained CRS activity. Unexpectedly, these splice donors were still utilized for splicing. These results indicate that the major HIV-1 splice donors can function as CRS and function to negatively regulate the cytoplasmic accumulation of HIV-1 RNAs in COS cells. © 1997 Academic Press

### INTRODUCTION

The expression of HIV-1 structural proteins is regulated at the level of splicing, transport, stability, and translation by the Rev protein (Feinberg *et al.*, 1986; Sodroski *et al.*, 1986; Malim *et al.*, 1988; Felber *et al.*, 1989; Hadzopoulou *et al.*, 1989; Hammarskjold *et al.*, 1989; Arrigo and Chen, 1991; Cochrane *et al.*, 1991; Lawrence *et al.*, 1991; D'Agostino *et al.*, 1992; Schwartz *et al.*, 1992). The Rev protein binds to an RNA binding site termed the Rev-responsive element (RRE) present within the RNAs that it regulates (Rosen *et al.*, 1988; Hadzopoulou *et al.*, 1989; Malim *et al.*, 1989). This regulation may involve alteration of the cellular pathway of the RNA (Fischer *et al.*, 1995). The Rev-dependent RNAs require Rev for protein expression and therefore must contain negative sequences, the effect of which is overcome by the Rev protein (for review see Dayton, 1996). The Rev-independent *tat*, *rev*, and *nef* genes presumably do not contain these sequences. These negative sequences have been termed *cis*-acting repressor sequences (CRS) and have been characterized in a variety of experimental systems. Splice sites have been implicated in Rev regulation by several groups. Introduction of sequences containing the HIV-1 *tat* 3' splice donor or splice acceptor sequences (i.e., those splice sites that join the Tat coding exons) into a construct containing a  $\beta$ -globin intron conferred Rev-responsiveness to the construct (Chang and Sharp, 1989). Rev-responsiveness could also be conferred by mutation of

the  $\beta$ -globin splice site consensus sequence within this construct. The CRS potential of splice donor sequences has been demonstrated by the Rev-responsiveness of papillomavirus constructs containing a splice donor in the 3' untranslated region (Barksdale and Baker, 1995). The major 5' splice donor of equine infectious anemia virus functions as a CRS within EIAV Gag-producing constructs, although it functions in a location-dependent manner (Tan *et al.*, 1996). Within HIV-1 Env-expressing constructs, splice sites and U1 interaction have been shown to be important for the regulation of Env expression by Rev (Lu *et al.*, 1990). Furthermore, the expression of Env could be made Rev-independent by placing a complete intron upstream of *env* and removing known splice donor sequences (Hammarskjold *et al.*, 1994).

Other sequences within HIV-1, distinct from splice sites, have also been shown to exhibit CRS activity. The fusion of various fragments of the *gag*, *pol*, and *env* genes to a CAT reporter construct resulted in Rev-responsiveness (Rosen *et al.*, 1988; Cochrane *et al.*, 1991). The CRS within *pol* elicited an orientation-dependent 30-fold negative effect on CAT activity that could be rescued by Rev and the RRE. This CRS is located within HIV-1 sequences that are not known to contain any splice sites. The effect appeared to be at the mRNA utilization level in that the subcellular distribution of RNA was not substantially affected. Fusion of regions of both *gag* and *pol* to reporter constructs also demonstrated at least two orientation-dependent CRS which functioned posttranscriptionally (Maldarelli *et al.*, 1991). These sequences do not contain known splice sites and were not substantially affected by the addition of splice sites to the constructs. Fusions of *gag* to a *tat*-expression vector were used to analyze CRS activity within *gag* (Schwartz *et al.*, 1992).

<sup>1</sup> To whom correspondence and reprint requests should be addressed at Department of Microbiology and Immunology, Medical University of South Carolina, Basic Science Building, Room 201, 171 Ashley Ave., Charleston, SC 29425-2230. Fax: (803) 792-2464. E mail: arrigosj@musc.edu.

A less than 300-base pair fragment of *gag* was capable of exerting an effect on the total RNA production, which was attributed to differences in RNA stability. Mutational inactivation of this CRS required 28-point mutations, demonstrating the complex nature of this CRS (Schwartz *et al.*, 1992). In the absence of known splice sites, *env* expression was shown to be Rev-dependent in mammalian cells using Env expression vectors (Nasioulas *et al.*, 1994). Localization of CRS within *env* identified many regions capable of exerting modest negative activity which together exerted more dramatic activity. Deletion of the RRE led to Rev-independent expression of Env from subgenomic *env* constructs, indicating that a CRS may overlap RRE sequences (Brighty and Rosenberg, 1994; Churchill *et al.*, 1996). The complexity of the interaction between the *gag* CRS and splicing was recently demonstrated (Mikaelian *et al.*, 1996). The *gag* CRS in conjunction with the RRE was capable of conferring Rev-responsiveness on a  $\beta$ -globin construct. Mutations in splice sites rendered these constructs Rev-independent. Therefore, it appears that CRS may function at a variety of levels to decrease the expression of RNAs containing these elements.

In this study, we have investigated the involvement of HIV-1 splice sites in Rev regulation through sequential mutagenesis of an infectious proviral clone. To attempt to avoid the complexities of analyzing multiple types of CRS, we initially deleted the majority of intronic sequences, retaining the splice sites necessary for the production of the multiply spliced RNAs. Using these constructs, we have analyzed the ratio of cytoplasmic to nuclear HIV-1 RNAs in the presence or absence of Rev or the RRE as an indicator of CRS activity.

## MATERIALS AND METHODS

### Plasmid constructions

The BI construct was constructed from pYKJRCSE/EBV- (Arrigo and Chen, 1991), using DR mutagenesis (Gatlin *et al.*, 1995). *gag*, *pol*, *vif*, and part of *vpr* were deleted (nt 790–5710), leaving the major splice donor and Gag methionine intact. *env* and *vpu* were removed by deleting from just downstream of the 3' splice donor for *tat*, *rev*, and *nef* to just upstream of the most 3' splice acceptor (nt 6145–8307). The BI construct retains the splice sites for the generation of *tat* and is deleted for the majority of *tat* intronic sequences. Further details on the generation of the BI construct are available from the authors. The BIR construct was generated by insertion of an RRE-containing *Sac*I fragment into the *Xba*I site within the *nef* sequences of the BI construct (Campbell *et al.*, 1996). FI and FIR constructs were generated using DR mutagenesis. The second *tat/rev* intron was precisely deleted from the BI and BIR constructs using the following oligonucleotide primers: 5'-TGCTTTGGTAGAGAAACTTGATGAGTC-TGAC-3' and 5'-ACCCTCCTCCCAGCAACGAG-3'. This

deletion creates an in-frame fusion of the two *tat* coding exons. SI and SIR constructs were generated using DR mutagenesis. The first *tat* intron was precisely deleted from BI and BIR constructs using the following oligonucleotide primers: 5'-CAGTCGCCGCCCTCGCCTC-3' and 5'-AATTGGGTGTCGACATAGCAGAATAGGC-3'.

The SD2R construct was generated by restriction digestion of the SIR construct with *Bam*HI and religation. The SD1 construct was generated using PCR and contains sequences from nt 153–793, and nt 9036–9736 from pYKJRCSE, including the sequences encompassing the major 5' HIV-1 splice donor. These sequences were cloned into pGem2 (Promega). Further details on the generation of the SD1 construct are available from the authors. The SD1R construct was generated by insertion of the RRE-containing *Spel* fragment (Campbell *et al.*, 1996) into the SD1 construct. The NI construct was generated by the cloning of a PCR fragment into the SD1 construct. A 160-bp fragment was amplified using the BI construct as a template and the oligonucleotide primers: 5'-ACA-ACCATGGAGGCTAGCTAGGGAACCCACTG-3' and 5'-AACAGAATTCTCGCTTCCGGTCCCTGTTC-3'. PCR conditions were as previously described (Arrigo *et al.*, 1992). The PCR product and the SD1 construct were digested with *Ncol* and *Eco*RI. The PCR product was inserted into the SD1 construct to generate NI using standard molecular techniques. The NI construct contains a deletion of almost all sequences between the splice donor and the polyadenylation signal, retaining 78 base pairs between these two elements.  $\Delta$ NI was generated from the NI construct using DR mutagenesis and the following oligonucleotide primers: 5'-AGAACCGCGGACGCCGAAATTTG-ACTAGCGGAGGC-3' and  $\Delta$ SD1A, 5'-ACTACCGCGGTCGCGCCGCCCTCGCCTTGC-3'. This results in the deletion of the consensus splice donor site, TGGTGAGT, from the NI construct and the insertion of the sequence CCGCGG.

### Cell culture and transfection

COS cells were maintained in Iscove's medium supplemented with 10% fetal calf serum. Transfections were performed as described previously (Arrigo *et al.*, 1989). A total of 50  $\mu$ g of plasmid DNA was electroporated for each sample. Cotransfections were performed with the indicated construct with or without the addition of pSVtat and/or pCMV Rev (Lewis *et al.*, 1990). A filler construct (pcDNA3, Invitrogen) was used to normalize the total amount of DNA to 50  $\mu$ g. A confluent T175-cm<sup>2</sup> flask of cells was used for approximately six transfections. RNA was harvested approximately 40 hr postelectroporation.

### RNA fractionation and analysis

Cells were fractionated as previously described (Favarro and Arrigo, 1997). Isolation of RNA and RT-PCR was performed as previously described (Arrigo *et al.*, 1989, 1990;

Arrigo and Chen, 1991). RT-PCR was performed for 20 cycles of amplification for all RNAs except that of the total RNA produced by NI and  $\Delta$ NI, which was amplified for 15 cycles. Most of the oligonucleotide primers used for detection have been previously described (Arrigo *et al.*, 1989, 1990; Arrigo and Chen, 1991). The unspliced RNAs produced by the BI/BIR, FI/FIR, SD1/SD1R, and NI/ $\Delta$ NI constructs were detected with the LA8/Gag-Nco oligonucleotide primers. The unspliced RNAs produced by the SI/SIR and SD2/SD2R constructs were detected with the LA45/AA821 oligonucleotide primers. The spliced RNAs produced by the BI/BIR and SI/SIR constructs were detected with the LA45/LA41 oligonucleotide primers. The spliced RNAs produced by the FI/FIR constructs were detected with the LA8/LA23 oligonucleotide primers. Oligonucleotide primers LA23 (5'-GCCTATTCTGCTATGTCGAC-CCC-3'; nt5815-5792) and Gag-Nco (5'-CGCACCCAT-GGCTCTCCTCTAG-3'; nt797-775) are antisense primers homologous to sequences downstream of the most 5' *tat* splice acceptor and major 5' splice donor, respectively. Total RNA produced by the SD1R, SD2R, and NI/ $\Delta$ NI constructs was detected with an oligonucleotide pair located upstream of the major 5' splice donor: LDR1 (5'-GAACAG-GGACCGGAAAGCG-3'; nt644-662) and  $\alpha$ -LDR1 (5'-GCG-CGCTTCAGCAAGCCGAG-3'; nt716-697).

Quantitation of the ratio of cytoplasmic to nuclear RNA was performed by scanning autoradiographs with a Lacie DTP Silverscan III scanner. Individual bands were quantitated with NIH Image 1.59. Analysis was performed on a Macintosh 7100/80 computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available from the Internet by anonymous FTP from zippy.nimh.nih.gov or on floppy disk from the National Technical Information Service, Springfield, VA, Part No. PB95-500195GEI). The cytoplasmic fractions (Cyto 1 and Cyto 2) were added together and compared to the nuclear fraction to determine the ratio of cytoplasmic to nuclear RNA. The level of nuclear RNA was arbitrarily set to a level of 1.

## RESULTS

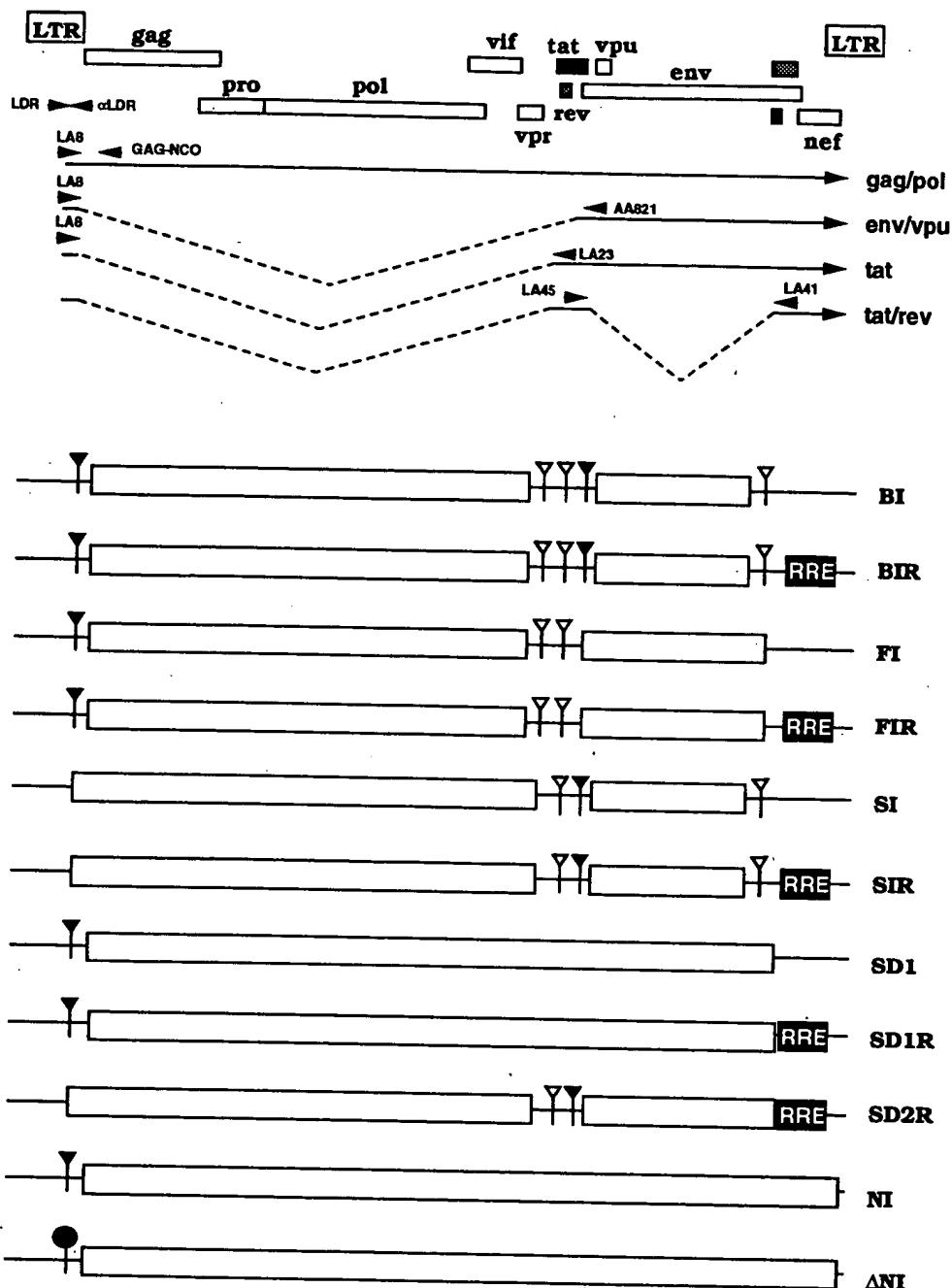
### Deletion of the majority of intronic sequences from HIV-1 does not eliminate CRS activity

CRS activity has been attributed to intronic sequences within *gag*, *pol*, and *env*. Starting with an infectious clone of HIV-1, the majority of intronic sequences were deleted. These deletions remove most of the sequences known to contain CRS activity. The resultant construct, BI, retains minimal amounts of both HIV-1 *tat* introns (Fig. 1). The BI construct retains 145 nucleotides of 5' and 150 nucleotides of 3' intronic sequences. The RNA produced by the BI construct should retain the ability to splice both of these minimal introns and should produce all three classes of HIV-1 RNA (unspliced, partially spliced, and fully spliced). In addition, this construct should be capable

of producing the Tat and Rev regulatory proteins. This construct did not contain the RRE, which has been shown to exhibit CRS activity in some experimental systems (Brighty and Rosenberg, 1994; Churchill *et al.*, 1996). The RRE was inserted into the *nef* gene of this construct to generate the construct BIR (Fig. 1). The presence of the RRE at this site has been previously shown to function in a similar manner to the normal location of the RRE (Campbell *et al.*, 1996). Since the BIR construct both produces Rev and contains the RRE, comparison of the distributions of RNAs produced by this construct with the distributions of RNAs produced by the BI construct should provide a good indication of the presence of CRS activity in the BI construct. The BI and BIR constructs were analyzed by transient transfection into COS cells. The cells were separated into three subcellular fractions (Favarro and Arrigo, 1997). RNA was isolated and analyzed using a quantitative RT-PCR procedure (Arrigo *et al.*, 1989). The results are shown in Fig. 2. The autoradiographs were quantitated to determine the ratio of cytoplasmic to nuclear RNA (C:N). A ratio of 1:1 indicates that 50% of the RNA was found in the cytoplasmic fractions and 50% was found in the nuclear fraction. In the absence of the RRE, the unspliced RNA produced by the BI construct had a predominantly nuclear distribution, as evidenced by a C:N ratio of 0.2:1. In the presence of the RRE, the unspliced RNA produced by the BIR construct had a predominantly cytoplasmic distribution, as evidenced by a C:N ratio of 4.5:1. The fully spliced RNA accumulated predominantly in the cytoplasm, regardless of the presence or absence of the RRE. The ratios of cytoplasmic to nuclear spliced RNA produced by the BI and BIR constructs were 20:1 and 16:1, respectively. The difference in the C:N ratio of unspliced RNA produced by the BI and BIR constructs indicated that the unspliced RNA produced by the BI construct retained substantial levels of CRS activity.

### Presence of a minimal intron maintains CRS activity

The unspliced RNA produced by the BI construct contained CRS activity, even though this construct was deleted for the majority of HIV-1 intronic sequences. Therefore, we wished to independently examine constructs containing each of the minimal *tat* introns for the retention of CRS activity within the unspliced RNA. To independently examine the first *tat* intron, a construct which contained sequences encoding the first minimal *tat* intron was generated (FI; Fig. 1). This construct was generated by precisely deleting sequences encoding the second *tat* intron from the BI construct, conserving *tat* and *rev* reading frames. The FI construct should produce an unspliced RNA and a spliced RNA encoding Tat and Rev proteins. The FIR construct was generated through insertion of an RRE into the *nef* gene of the FI construct (Fig. 1). Both FI and FIR constructs were transfected into COS cells and assessed by RT-PCR analysis of fractionated



**FIG. 1.** Constructs used to localize CRS activity in HIV-1. Schematic diagram of HIV-1 genome, HIV-1 RNAs, and constructs generated. Splice donors are represented as closed triangles and splice acceptors are represented as open triangles. Deletions are represented as open boxes. The closed circle represents the mutated splice donor site. The location of the RRE in the appropriate constructs is shown. Oligonucleotide primers used for detection of various RNAs are depicted as arrows above the RNAs. LDR1 and  $\alpha$ LDR1 are homologous to sequences in the RNA leader sequence present in all RNAs. LA8 is just upstream and Gag-Nco is just downstream of the major 5' splice donor. AA821 is just downstream of the env splice acceptor. LA23 is just downstream of the 5' tat splice acceptor. LA45 is just upstream of the major 3' splice donor and LA41 is just downstream of the 3' tat/rev splice acceptor.

RNAs (Fig. 3). In the absence of the RRE, the unspliced RNA produced by the FI construct had a predominantly nuclear distribution, as evidenced by a C:N ratio of 0.4:1. In the presence of the RRE, the unspliced RNA produced by the FIR construct had a predominantly cytoplasmic distribution, as evidenced by a C:N ratio of 2.0:1. The fully spliced RNA accumulated predominantly in the cyto-

plasm, regardless of the presence or absence of the RRE. The ratios of cytoplasmic to nuclear spliced RNA produced by the FI and FIR constructs were 4.4:1 and 3.8:1, respectively. The difference in the C:N ratio of unspliced RNA produced by the FI and FIR constructs indicated that the unspliced RNA produced by the FI construct retained substantial levels of CRS activity.

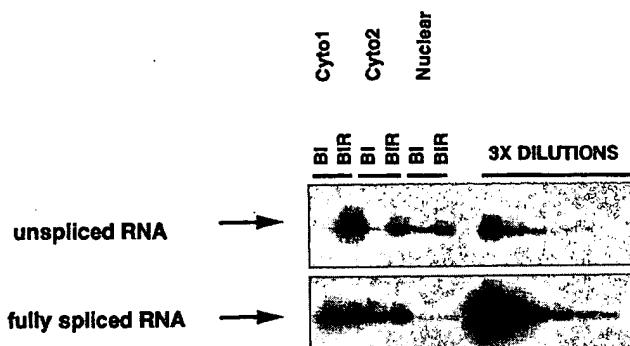


FIG. 2. Effect of deletion of the majority of intronic sequences on CRS activity. Results of RT-PCR analysis, using fractionated RNAs (Cyto 1, Cyto 2, and Nuclear) from COS cells transfected with 50  $\mu$ g of the indicated constructs. Cyto 1 and Cyto 2 fractions were generated using a buffer containing 0.05% NP-40 and 0.65% NP-40, respectively. The remaining pellet was designated the Nuclear fraction. Detection of unspliced and fully spliced RNAs is shown. Threefold dilutions of RNA from transfected cells were assayed to demonstrate the quantitative nature of this procedure.

To independently examine the second *tat* intron for CRS activity, a construct containing sequences encoding the second minimal *tat* intron was generated (SI; Fig. 1). This construct was generated by deletion of sequences encoding the first *tat* intron. The SI construct should produce an unspliced RNA encoding the Tat protein and a spliced RNA encoding Tat and Rev proteins. The SIR construct was generated through insertion of an RRE into the *nef* gene of the SI construct (Fig. 1). Both the SI and SIR constructs were transfected into COS cells and assessed for CRS activity by RT-PCR analysis of fractionated RNAs. The results are shown in Fig. 4. In the absence of the RRE, the unspliced RNA produced by the SI construct had a predominantly nuclear distribution, as evidenced by a C:N ratio of 0.8:1. In the presence of the RRE, the unspliced RNA produced by the SIR construct had a predominantly cytoplasmic distribution, as evidenced by a C:N ratio of 2.4:1. The fully spliced RNA accumulated predominantly in the cytoplasm, regardless of the presence or absence of the RRE. The ratios of cytoplasmic to nuclear spliced RNA produced by the SI and SIR constructs were 20:1 and 16:1, respectively. The difference in the C:N ratio of unspliced RNA produced by the SI and SIR constructs indicated that the unspliced RNA produced by the SI construct retained substantial levels of CRS activity.

#### Deletion of utilized splice acceptors does not eliminate CRS activity

Results had indicated that CRS activity was exhibited by RNAs containing either minimal *tat* intron. To further define the sequences responsible for the CRS activity within RNAs produced by the FI construct, the SD1 construct was generated. This construct retains the sequences encoding the 5' splice donor; however, se-

quences encoding all known splice acceptor sites have been removed (Fig. 1). The SD1 construct should produce only an unspliced RNA; however, splicing was detected in the RNA produced by this construct (see below). The SD1R construct was generated through insertion of an RRE into the SD1 construct (Fig. 1). SD1 and SD1R constructs were transfected into COS cells and assessed by RT-PCR analysis of fractionated RNAs using oligonucleotide primers that flanked the splice donor sequences to detect unspliced RNA. As these constructs do not produce Tat, all transfections included a Tat-producing plasmid. These constructs also do not produce Rev; therefore, transfections were performed in the presence or absence of a Rev-producing plasmid. The results are shown in Fig. 5, upper panel. The unspliced RNA produced by the SD1 construct, in the absence of Rev, was found predominantly in the nuclear fraction as evidenced by a C:N ratio of 0.6:1. The C:N ratio of unspliced RNA produced by the SD1 construct was not affected by the addition of Rev, as evidenced by a C:N ratio of 0.4:1. In the absence of Rev, the SD1R construct exhibited a similar RNA profile to that of the SD1 construct, as evidenced by a C:N ratio of 0.6:1. However, in the presence of Rev, the ratio of cytoplasmic to nuclear unspliced RNA produced by the SD1R construct was increased to a ratio of 5.5:1. These results indicated that the SD1R construct, containing the major HIV-1 5' splice donor, retained CRS activity within the unspliced RNA.

Although no known splice acceptor sequences were present within the SD1R construct, it was still possible that a cryptic splice acceptor might have been utilized in conjunction with the 5' splice donor. To determine whether the splice donor was utilized in the RNA produced by the SD1R construct, we further examined the RNA produced by this construct using oligonucleotide primers specific for sequences upstream of the splice donor to detect total RNA, both spliced and unspliced (Fig. 5, lower panel). In contrast to results using primers

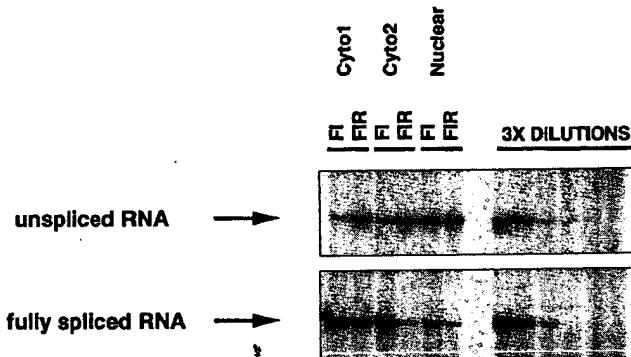


FIG. 3. Effect of the first minimal *tat* intron on CRS activity. Results of RT-PCR analysis, using fractionated RNAs (Cyto 1, Cyto 2, and Nuclear, as described in the legend to Fig. 2) from COS cells transfected with 50  $\mu$ g of the indicated constructs. Detection of unspliced and fully spliced RNAs is shown. Threefold dilutions of RNA from transfected cells were assayed to demonstrate the quantitative nature of this procedure.

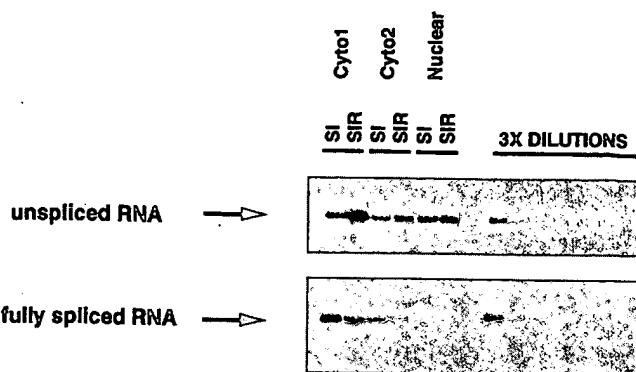


FIG. 4. Effect of the second minimal *tat* intron on CRS activity. Results of RT-PCR analysis, using fractionated RNAs (Cyto 1, Cyto 2, and Nuclear, as described in the legend to Fig. 2) from COS cells transfected with 50  $\mu$ g of the indicated constructs. Detection of unspliced and fully spliced RNAs is shown. Threefold dilutions of RNA from transfected cells were assayed to demonstrate the quantitative nature of this procedure.

that detected only unspliced RNA, the use of this set of primers indicated that Rev had little effect on the ratio of cytoplasmic to nuclear RNA produced by the SD1R construct. In fact, the bulk of the total RNA was found in the cytoplasmic fractions regardless of the presence or absence of Rev as evidenced by C:N ratios of 3.6:1 in the presence of Rev and 6.6:1 in the absence of Rev. This difference in C:N ratio was not seen in other experiments. These results indicated that the splice donor site within the RNA produced by the SD1R construct was utilized in combination with a cryptic splice acceptor site(s).

To better elucidate the CRS activity present within the unspliced RNA produced by the SI construct, the SD2R construct was generated. This construct retains sequences encoding the major 3' HIV-1 splice donor. Although the sequences encoding the *env/nef* splice acceptor are retained, they are positioned upstream of the 3' splice donor. The SD2R construct should produce only an unspliced RNA encoding Tat; however, splicing was detected in the RNA produced by this construct (see below). The SD2R construct was transfected into COS cells and assessed by RT-PCR analysis of fractionated RNAs using oligonucleotide primers that flanked the splice donor sequences to detect unspliced RNA. As this construct does not produce Rev, transfactions were performed in the presence or absence of a Rev-producing plasmid. The results are shown in Fig. 6. In the absence of Rev, the unspliced RNA produced by the SD2R construct had a C:N ratio of 0.5:1. However, in the presence of the Rev, the ratio of cytoplasmic to nuclear unspliced RNA was 2.8:1. These results indicated that the unspliced RNA produced by the SD2R construct retained CRS activity.

The RNA produced by the SD2R construct was further analyzed using oligonucleotides directed against sequences upstream of the splice donor to detect total RNA produced by the construct (Fig. 6). In contrast to

results using primers that detected only unspliced RNA, the use of these primers indicated that Rev had little effect on the distribution of RNA produced by the SD2R construct as evidenced by C:N ratios of 3.5:1 in the absence of Rev and 4.4:1 in the absence of Rev. These results indicated that the splice donor site within the SD2R construct was utilized in combination with a cryptic splice acceptor site(s).

#### An isolated splice donor functions as a CRS

Results obtained using the SD1R and SD2R constructs indicated that, in both cases, the splice donors were being utilized in conjunction with cryptic splice acceptors. The NI construct was generated through deletion of the bulk of sequences between the splice donor and the polyadenylation signal, leaving only 78 base pairs between these elements. Due to the lack of sequences downstream of the splice donor, we anticipated that the RNA produced by the NI construct would be extremely limited in its utilization of the major 5' splice donor. To elucidate the role of the major 5' splice donor in the manifestation of CRS activity, site-directed mutagenesis of this site within the NI construct was performed. The consensus splice donor sequence within the NI construct was mutated to generate the  $\Delta$ NI construct (Fig. 1). NI and  $\Delta$ NI constructs were transfected into COS cells in duplicate. Since these constructs do not produce Tat, all transfactions included a Tat-producing plasmid. Fractionated RNAs were assessed by RT-PCR analysis using oligonucleotide primers that

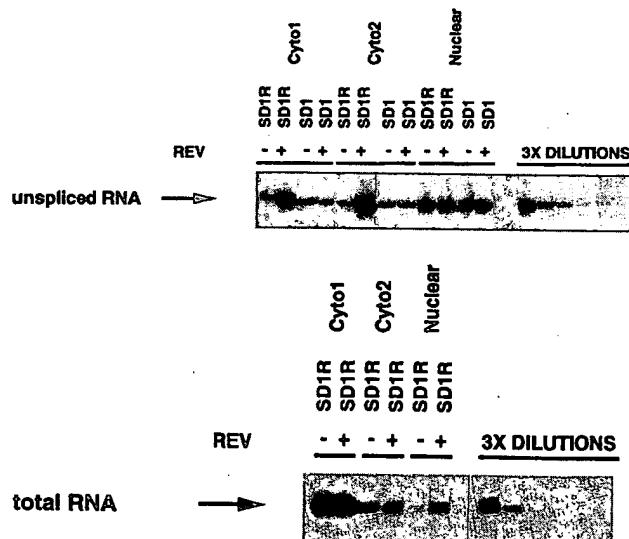


FIG. 5. Effect of deletion of known splice acceptors and retention of the major 5' splice donor on CRS activity. Results of RT-PCR analysis, using fractionated RNAs (Cyto 1, Cyto 2, and Nuclear, as described in the legend to Fig. 2) from COS cells transfected with 30  $\mu$ g of the indicated constructs. Detection of unspliced (upper panel) and total (lower panel) RNAs is shown. Threefold dilutions of RNA from transfected cells were assayed to demonstrate the quantitative nature of this procedure. All transfactions also contained 10  $\mu$ g of a Tat-producing construct. The presence or absence of 10  $\mu$ g of a Rev-producing construct is indicated (+ or -).

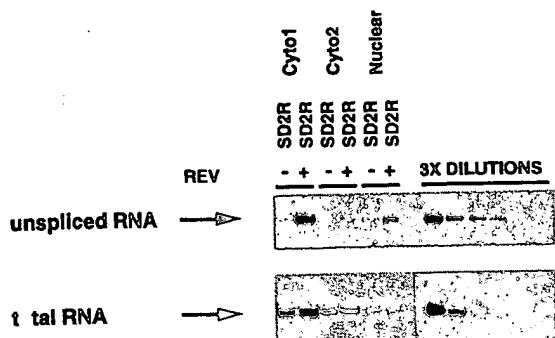


FIG. 6. Effect of deletion of known downstream splice acceptors and retention of the major 3' splice donor on CRS activity. Results of RT-PCR analysis, using fractionated RNAs (Cyto 1, Cyto 2, and Nuclear, as described in the legend to Fig. 2) from COS cells transfected with 25  $\mu$ g of the SD2R construct. Detection of unspliced and total RNAs is shown. Threefold dilutions of RNA from transfected cells were assayed to demonstrate the quantitative nature of this procedure. The presence or absence of 25  $\mu$ g of a Rev-producing construct is indicated (+ or -).

flanked the splice donor sequences. The results are shown in Fig. 7. The unspliced RNA produced by the NI construct was detected predominantly in the nuclear fraction as evidenced by a C:N ratio of 0.2:1–0.3:1. Mutation of the splice donor site ( $\Delta$ NI) resulted in an increase in the ratio of cytoplasmic to nuclear unspliced RNA as evidenced by a C:N ratio of 1.0:1–1.3:1. These results indicated that the 5' splice donor was functioning as a CRS within the unspliced RNA produced by the NI construct. To determine the extent to which the 5' splice donor was utilized, the RNA produced by the NI and  $\Delta$ NI constructs was further analyzed using oligonucleotides directed against sequences upstream of the splice donor to detect total RNA, both spliced and unspliced (Fig. 7). The ratio of cytoplasmic to nuclear total RNA produced by the NI construct was altered in that the C:N ratio was increased to 1.0:1–1.5:1. These results indicated that splicing was not completely eliminated within the RNA produced by the NI construct.

## DISCUSSION

CRS have been identified throughout the intronic sequences of HIV-1, in *gag*, *pol*, and *env* (Rosen *et al.*, 1988; Cochrane *et al.*, 1991; Maldarelli *et al.*, 1991; Schwartz *et al.*, 1992; Brighty and Rosenberg, 1994; Nasioulas *et al.*, 1994). In addition, splice sites have been implicated in HIV-1 Rev function (Chang and Sharp, 1989; Hammar-skjold *et al.*, 1994; Barksdale and Baker, 1995; Mikaelian *et al.*, 1996; Tan *et al.*, 1996). In this report, we have investigated the CRS activity of HIV-1 splice sites through sequential mutagenesis of a proviral clone. We have found that, in the absence of the majority of HIV-1 intronic sequences, Rev-responsiveness was still maintained. It is possible that an important component of CRS activity has been lost through deletion of these intronic sequences. The intronic sequences may still have a significant impact on CRS activity within the virus.

Unspliced RNAs containing either the first or second *tat* intron exhibited CRS activity. Removal of known splice acceptors did not eliminate the CRS activity present within the RNAs. These RNAs contained either the major 5' or 3' HIV-1 splice donor site. In the absence of any known downstream splice acceptors, these splice donors were still efficiently utilized, in conjunction with cryptic splice acceptors. Deletion of nearly all sequences between the major 5' HIV-1 splice donor and the polyadenylation site appeared to reduce, but did not eliminate, the utilization of the 5' major splice donor. Although deletion of these sequences did not relieve the CRS activity within the unspliced RNA, mutation of the splice donor did relieve CRS activity. Therefore, the major HIV-1 5' splice donor could function as a CRS, even in the absence of efficient splicing. These results clearly demonstrated that the HIV-1 splice donor sites are intimately involved in the regulation of the cytoplasmic accumulation of viral RNA.

The mutation of the splice donor site within  $\Delta$ NI also led to an apparent decrease in the total level of RNA. This reduction may be due to a decrease in stability of the RNA caused by the removal of the splice donor. A similar effect on total RNA levels was previously seen when the major 3' HIV-1 splice donor was mutated within a subgenomic *env* construct (Lu *et al.*, 1990), although the effect seen in the previous report was more severe and led to an almost complete inability to detect any RNA produced from the construct.

Results from several other groups support the idea that an isolated splice donor might function as a CRS. Naturally occurring inhibitory 5' splice sites within the 3' untranslated region of papillomaviruses were shown to function as CRS in that Rev can counteract their effect (Barksdale and Baker, 1995). The major 5' splice donor of equine infectious anemia virus was demonstrated to function as a CRS (Tan *et al.*, 1996). A  $\beta$ -globin splice donor was shown to be capable of functioning as a CRS (Chang and Sharp, 1989; Hammar-skjold *et al.*, 1994; Mikaelian *et al.*, 1996). The HIV-1 *tev* splice donor, located

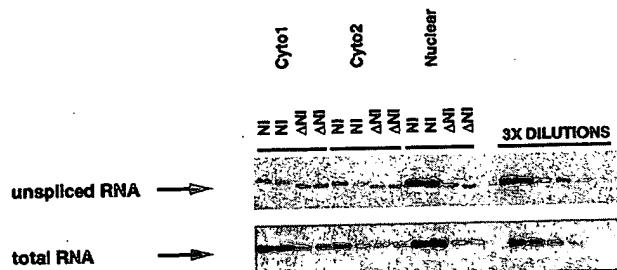


FIG. 7. The major 5' HIV-1 splice donor functions as a CRS. Results of RT-PCR analysis, using fractionated RNAs (Cyto 1, Cyto 2, and Nuclear, as described in the legend to Fig. 2) from COS cells transfected with 40  $\mu$ g of the indicated constructs in duplicate. Detection of unspliced and total RNAs is shown. Threefold dilutions of RNA from transfected cells were assayed to demonstrate the quantitative nature of this procedure. All transfections also contained 10  $\mu$ g of a Tat-producing construct.

within the HIV-1 *env* gene, also appears to function as a CRS (Hammarskjold *et al.*, 1994). The results presented in this paper indicate that authentic HIV-1 splice donors function as CRS to prevent the cytoplasmic accumulation of viral RNAs. The identification of utilized HIV-1 splice donor sites as CRS leaves open the possibility that cryptic HIV-1 splice donor sites might also function as CRS when expressed within the appropriate context.

It seems apparent that the control of HIV-1 RNA production involves a variety of regulatory activities. Splicing serves to downregulate the level of cytoplasmic unspliced and partially spliced RNAs. The splice donors themselves also serve to downregulate the cytoplasmic accumulation of those RNAs in which they are present. The intronic CRS may serve to retain RNA in the nucleus, decrease RNA stability, or decrease RNA cytoplasmic utilization (Rosen *et al.*, 1988; Cochrane *et al.*, 1991; Maldarelli *et al.*, 1991; Schwartz *et al.*, 1992; Brighty and Rosenberg, 1994; Nasioulas *et al.*, 1994). The combination of all these regulatory activities may synergize within the virus to prevent viral structural protein production. Rev may serve to overcome all of these levels of negative regulation and permit viral structural protein production.

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## Selectively Reduced *tat* mRNA Heralds the Decline in Productive Human Immunodeficiency Virus Type 1 Infection in Monocyte-Derived Macrophages

Secondo Sonza,<sup>1\*</sup> Helen P. Mutimer,<sup>1</sup> Kate O'Brien,<sup>1</sup> Philip Ellery,<sup>1</sup> Jane L. Howard,<sup>2</sup> Jonathan H. Axelrod,<sup>3</sup> Nicholas J. Deacon,<sup>4</sup> Suzanne M. Crowe,<sup>1</sup> and Damian F. J. Purcell<sup>2</sup>

*AIDS Pathogenesis Research Unit<sup>1</sup> and AIDS Molecular Biology Unit,<sup>4</sup> Macfarlane Burnet Institute for Medical Research and Public Health, Melbourne, Victoria 3004, and Department of Microbiology and Immunology, University of Melbourne, Parkville, Victoria 3052,<sup>2</sup> Australia, and Goldyne Savad Institute for Gene Therapy, Hadassah Medical Organization, Jerusalem 91120, Israel<sup>3</sup>*

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The transcription and splicing of human immunodeficiency virus type 1 (HIV-1) mRNA in primary blood monocyte-derived macrophages (MDM) and CD4<sup>+</sup> peripheral blood lymphocytes (PBL) were compared to determine whether any differences might account for the slower noncytopathic infection of cells of the macrophage lineage. The expression of regulatory mRNAs during acute infection of MDM was delayed by about 12 h compared to that of PBL. In each cell type, an increase in spliced viral mRNAs slightly preceded virus production from the culture. Following the peak of productive infection, there was a proportional decrease in the expression of all regulatory mRNAs detected in PBL. In MDM, a dramatic additional decrease specifically in the *tat* mRNA species heralded a reduction in virus production. This decline in *tat* mRNA was reflected by a concomitant decrease in Tat activity in the cells and occurred with the same kinetics irrespective of the age of the cells when infected. Addition of exogenous Tat protein elicited a burst of virus production from persistently infected MDM, suggesting that the decrease in virus production from the cultures is a consequence of the reduction in *tat* mRNA levels. Our results show that modulation of HIV-1 mRNAs in macrophages during long-term infection, which is dependent on the period of infection rather than cell differentiation or maturation, results in a selective reduction of Tat protein levels at the commencement of a persistent, less productive phase of infection. Determination of the mechanism of this mRNA modulation may lead to novel targets for control of replication in these important viral reservoirs.

The human immunodeficiency virus type 1 (HIV-1) genomic RNA undergoes a distinctly complex series of multiple splicing events to produce an array of 22 different 4.0-kb mRNAs for the Env, Vpu, Tat, Vpr, and Vif proteins and 22 different 1.8-kb mRNAs for the Tat, Rev, Vpr, and Nef proteins (38, 44, 47, 48). These seven viral proteins, encoded by more than 40 alternatively spliced mRNAs, regulate virus replication and important host cell functions and are crucial for HIV-1 infectivity and *in vivo* pathogenesis. Alterations to the balanced splicing of these HIV mRNAs can have a dramatic impact on viral infectivity and pathogenesis (44).

The large array of alternatively spliced HIV-1 mRNA primarily results from the differential use of six competing alternative 3' splice acceptor (SA) sites (A3, A4c, A4b, A4a, A5, and A7) (4, 28, 44, 47) and the use of two upstream noncoding exons (exons 2 and 3) that alter the 5' untranslated region (Fig. 1). RNA elements called exonic splice silencers (ESS) have been identified in the splice control regions of the genomic RNA that bind cellular heterogeneous nuclear ribonucleoproteins (hnRNPs) of the A, B, and H groups (9, 14, 30) and act to suppress the splicing of adjacent 3' SA sites, such as in the

second exon of *tat* (Fig. 1B, exon 4), the second *tat/rev* coding exon (exon 7), and noncoding exon 3 downstream of Vpr splice site A2 (2, 6, 57). Other elements called exonic splice enhancers (1, 57) bind serine/arginine-rich proteins such as ASF/SF2 and SC35 (59, 63) to enhance the splicing of neighboring exons, such as the third *tat* exon (Fig. 1B, exon 7). The high level of conservation of 5' SD and 3' SA sequences among different viral isolates of diverse origins suggests that complex splice regulation is important for efficient viral replication (45, 49).

The role of the complex HIV-1 RNA-processing control elements in modulating the course of HIV-1 infection of CD4<sup>+</sup> T lymphocytes and cells of the macrophage lineage is unclear. Many studies have demonstrated that blood monocytes and tissue macrophages display a characteristically slower, nonlytic, chronic course of infection, in contrast to primary CD4<sup>+</sup> T lymphocytes, in which HIV-1 infection is typically rapid, lytic, and highly productive of new virions (11, 41, 46). Some studies have suggested that the diverse outcomes of HIV-1 infection are due, in part, to different processing of viral RNA by these cells (32, 37, 43, 45).

However, most of these studies examining RNA splicing during HIV-1 replication have used T-cell lines, primary CD4<sup>+</sup> T lymphocytes, or monocytic cell lines in their analyses (32, 43, 45, 47). Relatively little is known about the control of RNA splicing in acutely or chronically infected primary cells of the macrophage lineage, although these cells are primary targets of

\* Corresponding author. Mailing address: Macfarlane Burnet Institute for Medical Research and Public Health, G.P.O. Box 2284, Melbourne, Victoria 3001, Australia. Phone: 61 3 9282 2173. Fax: 61 3 9282 2142. E-mail: sonza@burnet.edu.au.

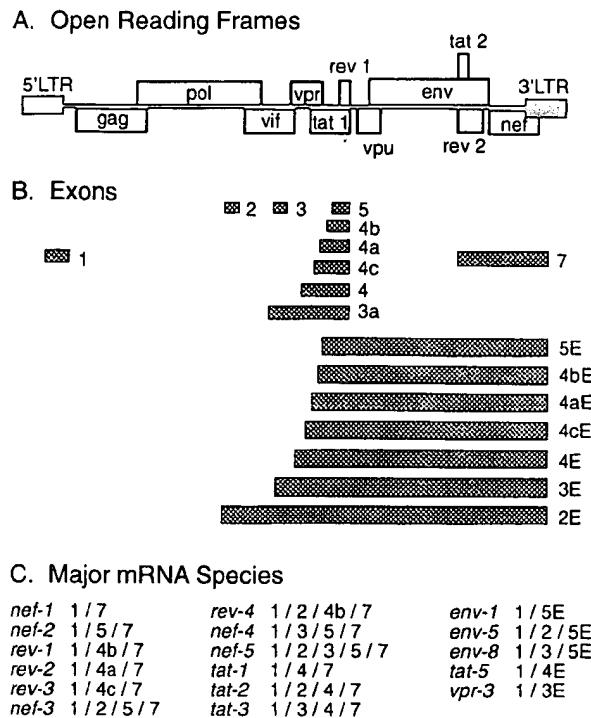


FIG. 1. Combinations of exons that are spliced from the HIV RNA genome join to form the alternatively spliced mRNAs of HIV-1. (A) Schematic representation of the HIV-1 genome. Open boxes show locations of the open reading frames that encode the viral proteins. (B) HIV-1 exons are generated by the use of different combinations of SAs and splice donors during RNA splicing. The exons, represented as bars, are numbered as described by Muesing et al. (38). (C) The exon contents of the major 1.8- and 4.0-kb classes of spliced transcripts detected in MDM and PBL are shown in abbreviated form, e.g., *nef-1* combines exons 1 and 7 and is annotated as 1/7.

HIV-1 infection and are important in the initial transmission event and in maintaining the infection (25, 54, 55). Freshly isolated monocytes are poorly susceptible to infection *in vitro* but become increasingly susceptible during differentiation in culture over 5 to 7 days to a macrophage phenotype (52, 53), at least in part because of the increased expression of the CCR5 coreceptor (40). Because of the inherent difficulties of obtaining tissue macrophages for study, these *in vitro*-derived macrophages are often used as a model of their *in vivo* counterparts. In this study, we examined whether modulation of the large array of alternatively spliced HIV-1 regulatory transcripts correlates with the differential viral replication and expression observed following *in vitro* infection of primary monocyte-derived macrophages (MDM) compared to peripheral blood lymphocytes (PBL). This work increases our understanding of HIV-1 infection in macrophages and has the potential to lead to novel therapeutic targets for the control of replication in these important reservoirs.

## MATERIALS AND METHODS

**Isolation of lymphocytes and monocytes from blood.** PBL and monocytes were isolated from HIV-1-seronegative blood cell packs (Red Cross Blood Bank, Melbourne, Australia) by density gradient centrifugation and plastic adherence in 175-cm<sup>2</sup> petri dishes as previously described (52). The nonadherent PBL were

resuspended in RPMI 1640 medium (Gibco BRL, Grand Island, N.Y.) with 10% fetal calf serum, 2 mM L-glutamine (ICN Biomedicals Inc., Costa Mesa, Calif.), and 50 µg of gentamicin per ml (RF10) and then stimulated with phytohemagglutinin (2.5 µg/ml; Wellcome Diagnostics, Dartford, United Kingdom) for 2 to 3 days prior to infection with HIV-1. Infected PBL were maintained in flasks in RF10 with the addition of interleukin-2 (10 U/ml; Roche, Castle Hill, Australia), and the medium was changed twice weekly. The monocytes were detached from the petri dishes and usually maintained adherent in 24-well plates (Nunc, Naperville, Ill.) in Iscove's medium (Gibco BRL) supplemented with 10% AB/Rh<sup>+</sup> human serum, glutamine, and gentamicin (IH10) at a concentration of 10<sup>6</sup> monocytes/well. They were then washed thoroughly with phosphate-buffered saline (PBS) and infected 5 to 7 days after isolation, by which time they had differentiated to a macrophage phenotype and were maximally permissive to infection with R5 strains of HIV-1. In one experiment, monocyte-derived macrophages were infected at approximately weekly intervals until 4 weeks after isolation. MDM cultures were given weekly medium changes and maintained under endotoxin-free conditions throughout.

**Viral strains and infection assays.** Replicate wells of  $10^6$  MDM or cultures of PBL at  $2 \times 10^6$  cells/ml were infected for kinetic studies with the prototype R5 strain, HIV-1<sub>Ba-L</sub> (obtained from the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health; high-titer virus stocks produced in peripheral blood mononuclear cells [PBMC] [24]), or with a recombinant R5 strain, HIV-1<sub>NL(AD8)</sub> [supernatant from SW480 cells transfected with pNL(AD8), a stable, full-length molecular clone of pNL4-3 in which the envelope gene has been replaced with that from the macrophage-tropic ADA isolate (58)]. Cells were incubated with virus for 2 h at a multiplicity of infection (MOI) of 0.1 to 1 infectious particle per cell (assessed by determining the 50% tissue culture-infective dose in MDM), followed by thorough washing with PBS. Virus production was monitored by assaying for virion-associated reverse transcriptase (RT) activity by using [ $\alpha$ -<sup>32</sup>P]TTP incorporation into an oligo(dT)-poly(A) template (micro-RT assay) (60). To one long-term-infected culture, Tat protein (obtained through the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health, and contributed by J. Brady [7]), 13-phorbol-12-myristate acetate (PMA; Sigma Chemical Co., St. Louis, Mo.), or tumor necrosis factor alpha (TNF- $\alpha$ ) was added in an attempt to reactivate virus production. Tat (200 ng/well in the presence of 100  $\mu$ g of protamine sulfate per ml to enhance uptake [19]), PMA (100 ng/ml), and TNF- $\alpha$  (100 ng/ml) were added to the culture medium 4 to 5 weeks after infection. Cells were then monitored for a further 2 weeks with a medium change but no replenishment of additives after the first week. Also, to cultures from four different donors, recombinant soluble CD4 (rsCD4; 50  $\mu$ g/ml; a generous gift from Glaxo-SmithKline, King of Prussia, Pa. [13, 53]) was added immediately after infection with DNase-treated Ba-L (10 U of RNase-free DNase [Roche] per ml of virus stock in 10 mM MgCl<sub>2</sub> for 30 min at room temperature) and HIV-1 replication was assessed by monitoring the accumulation of viral DNA by PCR as described previously (33, 54).

**Bioassay for HIV-1 Tat activity.** An assay system consisting of a set of two adenovirus-luciferase reporter vectors, of which one is responsive to HIV-1 Tat protein activity (Ad-HIVluc) and the other is a control vector that is not Tat responsive because of a deletion of the TAR site (Ad-HIVΔTARluc), was used to assess Tat activity in HIV-infected MDM and PBMC cultures (5). The vectors were grown and titrated in 293 cells as previously described (5), and then each was used at an equivalent MOI of 100 to superinfect triplicate wells of 96-well plates containing  $10^5$  MDM or PBMC from three separate donors at twice weekly intervals for 6 and 3 weeks, respectively, following HIV-1 infection. The cells were washed with PBS 48 h after adenovirus superinfection and then lysed in 20  $\mu$ l of cell culture lysis reagent (CCLB; Promega) per well and stored at  $-20^{\circ}\text{C}$  until analyzed for luciferase activity at the completion of the experiment. Luciferase activity was measured by mixing 10  $\mu$ l of cell lysate (undiluted for PBMC lysates and diluted 1:10 in CCLB for MDM lysates) with 50  $\mu$ l of luciferase assay reagent (Promega) and immediate detection of the emitted light in a Triathler luminometer (Perkin-Elmer, Branchburg, N.J.). The presence of Tat activity in this bioassay is indicated by threefold or greater luciferase activity in Ad-HIVluc-infected cells than in those infected at the same time point with Ad-HIVΔTARluc, which measures the background level of Tat-independent transactivation of the HIV-1 long terminal repeat (LTR) in the cells.

**PCR for luciferase DNA.** The above-described CCLB lysates (5  $\mu$ l of each replicate pooled) were digested with proteinase K (10 to 15  $\mu$ g; Roche) at 60°C for 1 h and then at 95°C for 10 min. The lysates were then clarified by centrifugation at 13,000  $\times g$  for 2 min, and 5  $\mu$ l was used in a PCR with primers specific for the firefly (*Photinus pyralis*) luciferase (*luc*) gene (*luc-5'*, GAAGCTTGTG GATCTGGATACCC, positions 1621 to 1642; *luc-3'*, ACCTATGTCTCCAGAA

TGTAGCC, positions 1780 to 1758 [15]). The primers were used at a final concentration of 0.4  $\mu$ M in reaction mixtures containing 1.5 mM MgCl<sub>2</sub>, 0.2 mM deoxynucleoside triphosphates, and 1 U of *Taq* polymerase for 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The 160-bp PCR products were analyzed by electrophoresis in 2% agarose gels and stained with ethidium bromide, and band intensities upon UV illumination were compared by phosphorimaging (Fuji LAS1000).

**Preparation of mRNA and cDNA.** Poly(A)<sup>+</sup> RNA was purified at various time points postinfection with magnetic oligo(dT)<sub>25</sub> Dynabeads (Dynal, Oslo, Norway) at a concentration of 25  $\mu$ l of beads/10<sup>6</sup> cell equivalents. The cells were lysed, incubated for 10 min at room temperature with the oligo(dT)<sub>25</sub> beads, and washed in accordance with the manufacturer's instructions. The RNA was then reverse transcribed to cDNA with a 25- $\mu$ l reaction mixture containing the bead-mRNA complex, 1 $\times$  avian myeloblastosis virus RT buffer, 1 mM deoxynucleoside triphosphates, 1 U of RNasin per  $\mu$ l, and 1 U of avian myeloblastosis virus RT per  $\mu$ l (all from Promega Corp., Madison, Wis.). Samples were incubated for 1 h at 42°C, the RT mixture was removed, and the beads were resuspended in 10  $\mu$ l of elution solution (2 mM EDTA) before being heated to 95°C for 5 min to remove the mRNA. The cDNA libraries attached to the beads were stored in 10 mM Tris-HCl-1 mM EDTA, pH 7.6, at 4°C for use in subsequent reverse transcription-PCR analyses.

**Standardization and amplification of HIV-1 spliced mRNA.** To ensure a standard input of cDNA in HIV-specific PCRs, 10<sup>5</sup> cell equivalents of cDNA attached to beads was serially diluted to less than 1 cell equivalent and PCR amplified with primers specific for  $\beta$ -actin, BA1/4 [26]. PCR products (360 bp) were detected by electrophoresis in 2% agarose and ethidium bromide staining. Band intensities for all samples were compared by phosphorimaging as described above, and equivalent amounts of the cDNA were used in a subsequent PCR for viral RNA.

Following standardization of the cDNA, we performed semiquantitative analyses for the 1.8-kb class of HIV-1 RNA with 1  $\mu$ M primers Odp45 and Odp32 as described before [44] and for the 4.0-kb mRNA with primers Odp45 and Odp84 (5'-TCATTGCCACTGTCTTCTGCTCT-3') in a hot-start PCR (25 cycles of 95°C for 1.5 min, 55°C for 1 min, and 72°C for 2.5 min with a final 7 min at 72°C). One microliter of each amplification product was radiolabeled by performing a single round of PCR as before but with the addition of 10  $\mu$ Ci of [ $\alpha$ -<sup>32</sup>P]dCTP. The labeled samples were subsequently analyzed by electrophoresis through a 6% polyacrylamide-urea gel at sufficient wattage to maintain a temperature of 65°C. Bands were visualized by autoradiography and/or phosphorimaging (Fuji FLA2000). We have shown previously that this protocol preserves the relative proportions of the 1.8- and 4.0-kb classes of HIV-1 RNA [44].

## RESULTS

**Contribution of multiple rounds of HIV-1 replication to infection in primary MDM and PBL cultures.** Since the kinetic accumulation of spliced HIV-1 RNA might be confounded by asynchronous secondary infection, we used both a relatively high multiplicity of virus for infection (MOI of 1) and blocked subsequent rounds of infection in cultures from four donors with rsCD4. The degree of asynchronous infection of MDM was measured by comparing the relative abundance of *gag* viral DNA to that of cellular HLA-DQ- $\alpha$  DNA in the presence and absence of blocking concentrations of rsCD4. The amounts and ratios of *gag* and HLA-DQ- $\alpha$  DNA were similar in both treated and untreated MDM cultures and remained relatively constant from 48 h after infection (data not shown), demonstrating maximal synchronous infection of susceptible cells with minimal virus superinfection or spread after initial infection.

**MDM and PBL differ in the kinetic expression and splicing of HIV-1 mRNA during acute infection.** The splicing of HIV-1 mRNA during acute infection of primary PBL and MDM with Ba-L was investigated (Fig. 2). A 12-h delay in the first appearance of HIV-1 mRNA from MDM (24 h), compared to that of PBL (12 h; Fig. 2A), results from the delayed establishment of HIV-1 infection in the former cell type. Both

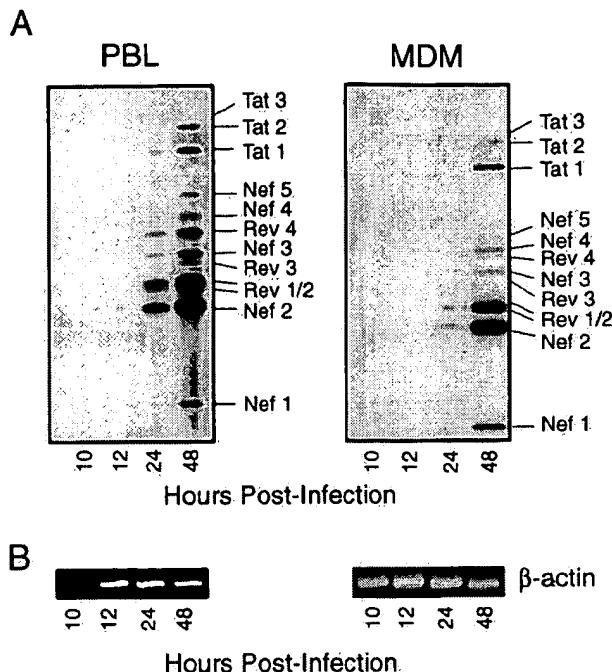


FIG. 2. The kinetic expression of alternatively spliced 1.8-kb viral mRNA during early HIV infection of PBL and MDM. Poly(A)<sup>+</sup> RNA was extracted from cells at 2-h intervals for the first 12 h and then at 24 and 48 h after infection with Ba-L. Semiquantitative PCR with primers Odp45 and Odp32 for multiply spliced transcripts (A) was performed on cDNA standardized by  $\beta$ -actin PCR (B). In our reverse transcription-PCR assay, the detection limits of 1.8- and 4.0-kb mRNA transcripts were 10<sup>2</sup> and 10<sup>3</sup> HIV-infected cells, respectively. The RNA patterns shown are representative of results of six experiments.

primary cell types expressed the same mRNA species. However, significant differences in the relative abundance of particular isoforms were observed between the cell types during the first 48 h. In MDM, *nef-1* (Fig. 1C, 1/7), *nef-2* (1/5/7), *rev-1* (1/4b/7), and *tat-1* (1/4/7) were the most abundant spliced isoforms of HIV-1 1.8-kb mRNA at 48 h (Fig. 2A, right side). *nef-2* and *rev-1* appeared simultaneously at 24 h postinfection, and the other *tat*, *rev*, and *nef* multiply spliced mRNAs (Fig. 1C) were detectable at 48 h postinfection. MDM expressed *tat-1* mRNA at a significantly higher level than the other *tat* mRNAs. In contrast, in the PBL cultures, only a subset of spliced HIV-1 mRNA species, *nef-2*, *rev-1*, and *rev-2* (1/4a/7), that predominate at 48 h were detected 12 h postinfection (Fig. 2A, left side). Other mRNAs prominent in PBL at 48 h postinfection, most notably, *nef-1*, *tat-1*, and *tat-2* (1/2/4/7), were proportionally underrepresented at earlier time points. PBL synthesized high levels of several other isoforms of *nef* and *rev* (i.e., *rev-2*, *rev-3*, *rev-4*, *nef-3*, *nef-4*, and *nef-5*) that were detected at relatively low levels in infected MDM. Similar mRNA profiles were found following infection of cells from six different donors.

**Alternative splicing of HIV mRNA during chronic infection in MDM and PBL.** We next examined the splicing of HIV-1 mRNA in primary PBL and MDM during chronic infection over a 3-week period (Fig. 3). Duplicate cultures of MDM or a single culture of PBL from each of two donors were infected

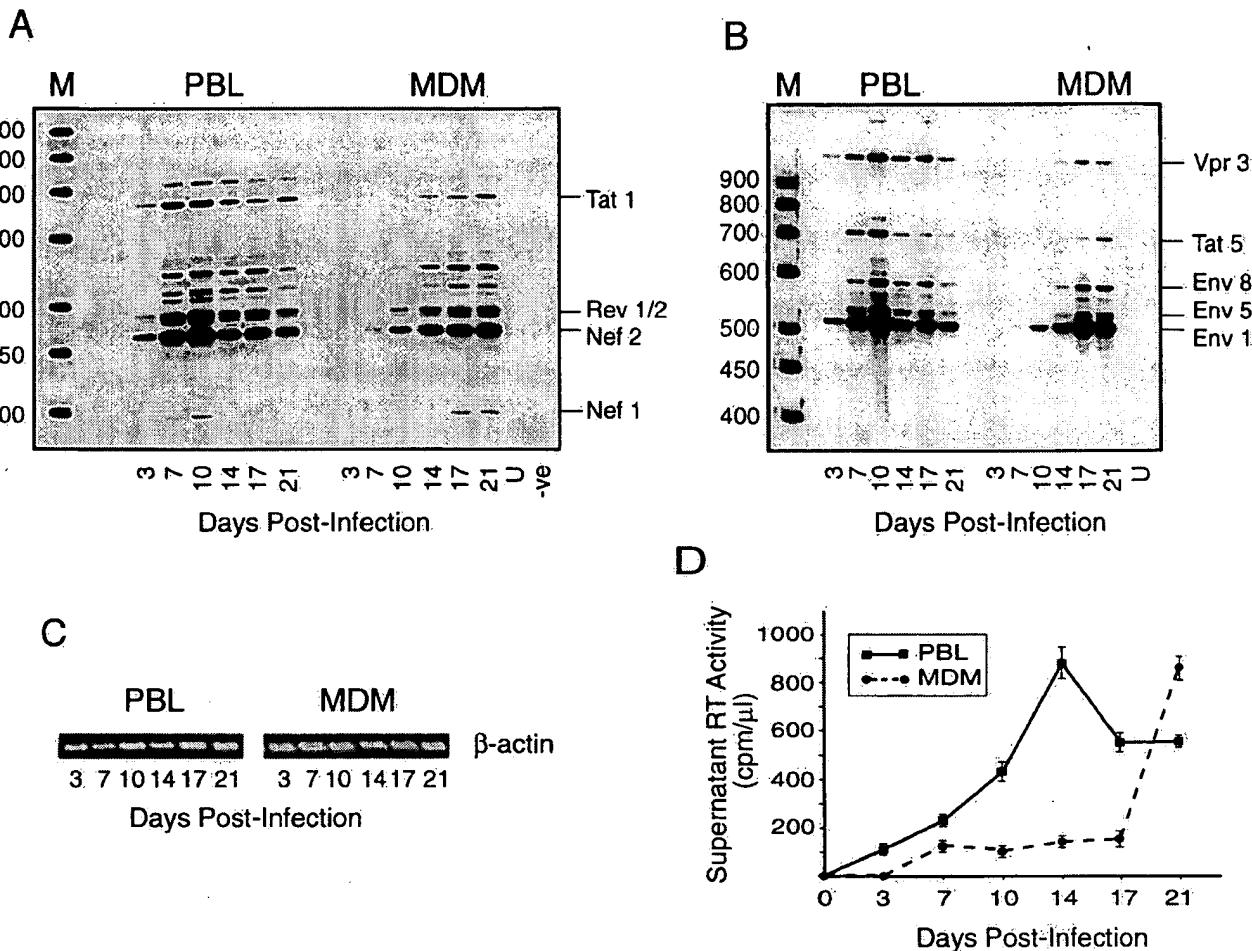


FIG. 3. Spliced HIV-1 mRNA accumulates in similar patterns in lymphocytes and macrophages. Duplicate cultures of MDM and a single culture of PBL were infected with Ba-L for sequential analysis of spliced HIV RNA. PBL were maintained at  $2 \times 10^6$  cells/ml, and RNA was extracted from  $5 \times 10^5$  cells that were removed from the PBL infection or from  $10^6$  replicate infected MDM at intervals of 3 or 4 days over a 3-week period. (A) Expression of 1.8-kb viral mRNA in PBL and MDM from the same donor infected with HIV-1<sub>Ba-L</sub>. Semiquantitative PCR with primers Odp45 and Odp32 was performed with standardized amounts of cDNA (C) attached to magnetic beads. (B) Viral 4.0-kb mRNA profile of the same samples as in panel A following amplification with primers Odp45 and Odp84. Note that the gel in panel B was exposed four times longer than that in panel A for detection of minor bands. Patterns shown for 1.8- and 4.0-kb RNAs were similar in two donor lymphocyte and monocyte populations. (D) Virus production from the cultures shown in panels A and B as assessed by supernatant RT activity. M, molecular size markers (sizes are in base pairs); U, uninfected-cell control; -ve, no-cDNA control.

for sequential analysis of spliced HIV-1 RNA at intervals of 3 or 4 days. Reverse transcription-PCR analysis of the different alternatively spliced 1.8- and 4.0-kb HIV-1 RNAs showed that the relative proportions of the various species were similar over this time (Fig. 3A and B), indicating that there was little difference between PBL and MDM in the cell-controlled splicing of HIV-1 RNA.

The kinetic expression of HIV-1 RNA differed between the MDM and PBL cultures. The production of spliced 1.8- and 4.0-kb HIV-1 RNAs in PBL (Fig. 3A and B, respectively) peaked at 7 to 10 days after infection (cell donor dependent) and then gradually declined in a uniform manner until day 21, by which time the viability of the lymphocytes had significantly diminished. The time of maximum RNA production in PBL typically preceded the peak of supernatant RT activity by 3 or 4 days (Fig. 3D). The decline in production of HIV-1 RNA

after 7 to 10 days in PBL resulted from a proportionate decrease in HIV-1-infected cells because of both their death and their failure to divide in the culture (42, 62). This is reflected in virus production from these cells, which declined by days 17 to 21 (Fig. 3D), although there was still significant expression of the standard pattern of alternatively spliced HIV-1 mRNAs at this time (Fig. 3A and 3B).

As observed in acute infection, HIV-1 mRNA accumulated with much slower kinetics in infected MDM than in PBL during chronic infection (Fig. 3A and B). In contrast to PBL, both the overall level of viral 1.8-kb mRNA and virus production in MDM continued to increase until day 21 (Fig. 3A and D). HIV-1 infection of MDM, which are nondividing, does not result in significant cell death (11). The slight lag in the detection and the relatively lower abundance of the 4.0-kb mRNA compared to the 1.8-kb RNA in MDM (four times longer

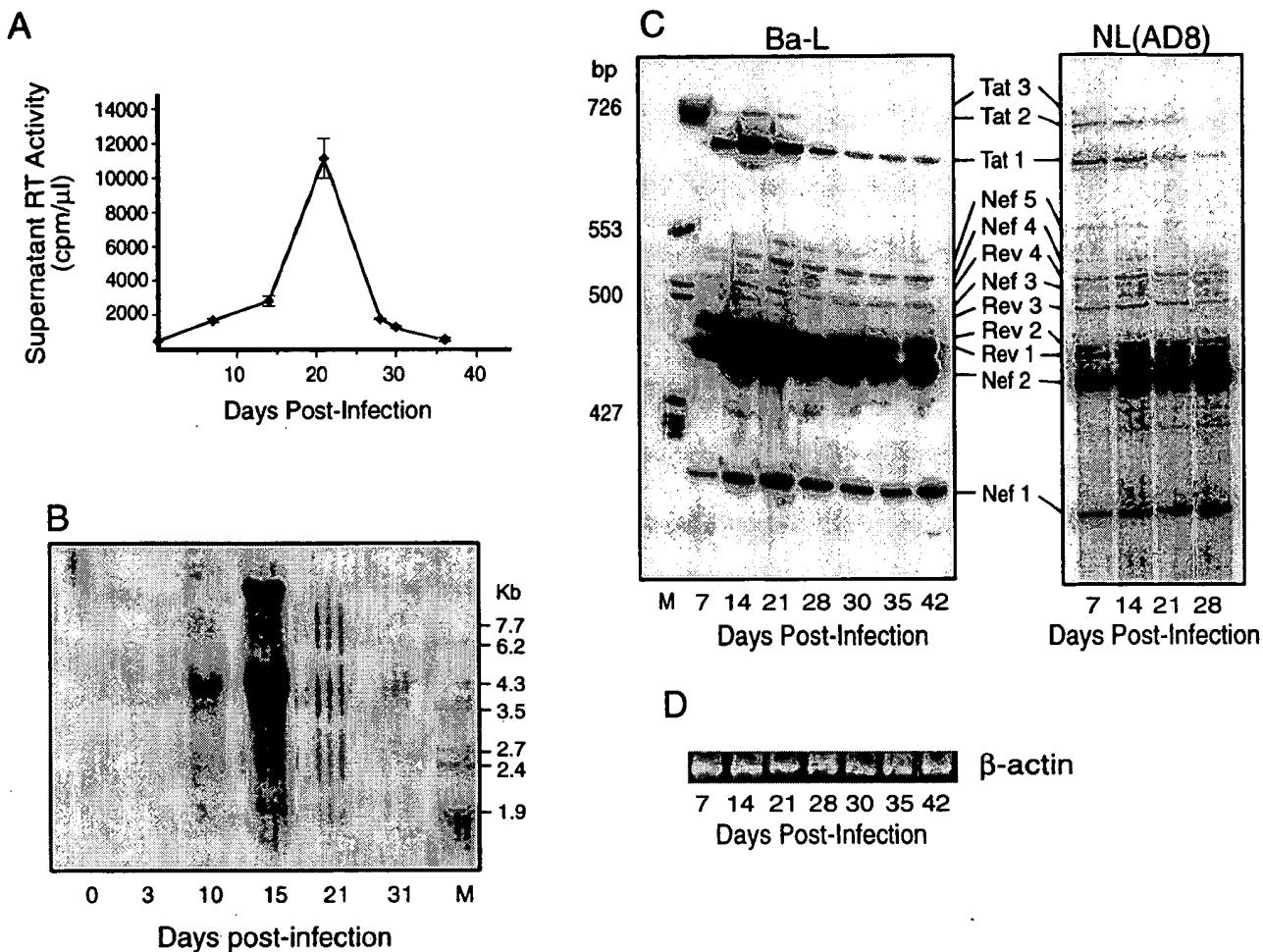


FIG. 4. Selective reduction in the abundance of HIV-1 *tat* mRNA marks the beginning of virus dormancy in MDM during long-term HIV-1 infection. MDM were infected with Ba-L after 5 days in culture, and samples for RNA and virus production were taken approximately weekly for 5 to 6 weeks. (A) Virus production was assessed by determining the virion-associated RT activity in culture supernatants. (B) Northern blot analysis of total RNA from a representative culture. Purified mRNAs from the same culture as in panel A were first standardized with  $\beta$ -actin PCR (D), and then equivalent amounts were amplified with primers Odp45 and Odp32 for multiply spliced viral transcripts (C, left side). A similar analysis was performed with MDM infected with NL(AD8) (C, right side). M, molecular size markers (kilobases or base pairs). Results similar to those shown were found for 10 donor MDM populations infected with Ba-L and several others with NL(AD8) and 676 (not shown).

exposure for Fig. 3B than for Fig. 3A) reflects the requirement for Rev prior to the cellular accumulation of the 4.0-kb mRNAs that contain the Rev-responsive element.

**Marked alteration of HIV-1 RNA profile as MDM enter a nonproductive phase during long-term infection.** We next examined viral RNA processing in MDM that were maintained in culture for 6 weeks following infection (Fig. 4). We sought to determine whether the diminished production of HIV-1 in MDM after a peak of infection at around 3 weeks was due to an alteration in the profile of spliced viral mRNA. MDM cultures infected with Ba-L produced little or no detectable virus in the supernatant after 28 days (Fig. 4A) and very little viral RNA as determined by Northern blotting of total cellular RNA (Fig. 4B). Reverse transcription-PCR analysis of the spliced HIV-1 RNA showed that the peak of RT activity coincided with, or slightly followed, the peak in abundance of viral RNA occurring between days 14 and 21 (Fig. 4A and C).

It was not until after the peak of productive infection that any variation in the RNA profile was observed. We found a significant decrease in the relative proportion of *tat*-specific mRNA at times later than 21 days, compared to that of all other alternatively spliced mRNA.

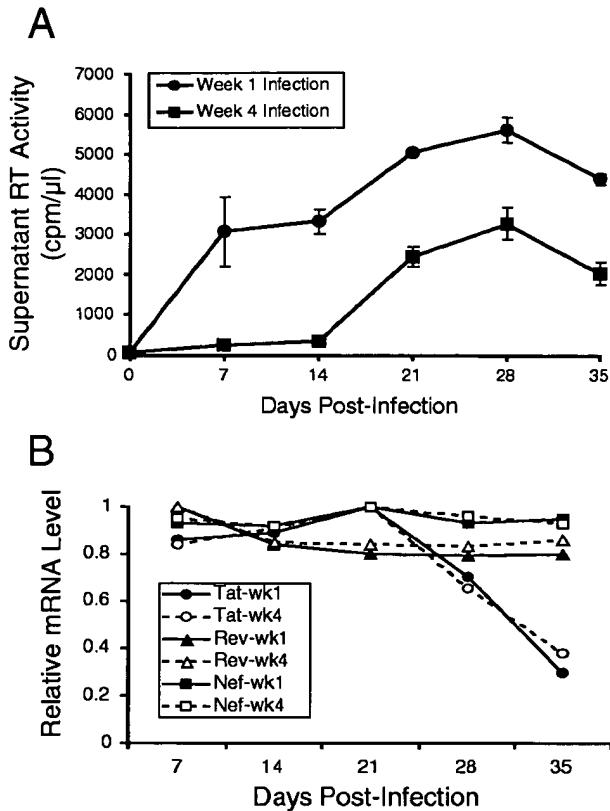
The most striking change in RNA processing during long-term infection of MDM was the dramatic decrease observed in the relative accumulation of *tat*-1. The *tat*-1 mRNA was one of the most abundant mRNA species at the peak of infection (Fig. 4C, days 14 to 21) but became a very minor species by the end of the culture period. The decline in *tat*-1 was coincident with the decrease in productive infection in MDM cultures and was in marked contrast to the continued abundance of the other major species, *nef*-1, *nef*-2, *rev*-1, and *rev*-2. The decline in each of the *tat* mRNA species indicates a selective decrease in the viral RNA spliced at SA3, located at the 5' end of exon 4. This exon contains the translation initiation site of Tat (Fig.

1). The selective decrease in *tat* mRNA species was observed during infection of each of 10 MDM donors with Ba-L and several more with NL(AD8) (Fig. 4C) and the primary isolate HIV-1<sub>676</sub> (52) (data not shown); however, the relative decrease in *tat* differed between donors, ranging from 60-fold (Fig. 4) to 10-fold (see Fig. 8). This selective decrease in *tat* species was not observed in lymphocytes in which, after the peak in RNA production at 7 to 10 days, all spliced transcripts declined proportionally until day 21 (Fig. 4A). As was found in the PBL cultures, the three-exon form of *nef* mRNA (*nef*-2) was the predominant viral mRNA expressed throughout the course of infection in MDM, along with the two three-exon forms of *rev* mRNA (*rev*-1 and *rev*-2). The two-exon *Nef* mRNA (*nef*-1) was not as prevalent as previously reported (45).

**Altered proportions of spliced viral mRNA in long-term-infected MDM is not dependent on the age of the cells.** The marked specific reduction in Tat-encoding mRNAs during long-term infection in MDM could conceivably be due to changes induced in the cells by long-term culture. To determine whether this is the case or whether it arises as a consequence of infection, MDM from the same donor were infected with Ba-L at approximately weekly intervals over a 4-week period following isolation of the cells from blood. Samples were then taken weekly for 5 weeks and analyzed for virion-associated RT production and viral mRNA expression as above.

While MDM maintained in culture for a month were still permissive to infection with HIV-1, the amount of virus produced at peak infection was smaller and the kinetics of replication were more delayed with increasing time in culture before infection (Fig. 5A). When mRNA expression of the major 1.8-kb viral mRNA species over the course of infection was compared between the cells infected at different times after isolation, only the *tat* mRNAs again showed a pattern that correlated with virus production from the cells, i.e., an initial increase followed by a dramatic decline approximately 1 week before virus production waned. The *tat* species (*tat*-1, -2, and -3) again decreased markedly over the final 2 weeks of culture relative to their peak expression level around 3 weeks after infection in this donor, irrespective of when the cells were infected (Fig. 5B). In contrast, the *rev* and *nef* mRNAs remained at similar relative proportions throughout the course of infection. The patterns of viral mRNA expression were remarkably similar regardless of how long after isolation the cells were infected, with only the overall abundance somewhat reduced with increasing time in culture before infection (not shown), correlating with virus production from the cells (Fig. 5A).

**Declining *tat* mRNA abundance results in dramatically lower Tat protein activity in long-term-infected MDM.** To determine if the observed decrease in *tat* mRNA expression after peak virus production in MDM resulted in a corresponding decrease in the level of Tat activity in these cells, monocytes from three different donors were cultured in 96-well plates and infected 5 to 7 days after isolation. Triplicate wells were then superinfected with recombinant adenovirus vectors containing the firefly luciferase gene under the control of either the wild-type HIV-1 LTR (Ad-HIVluc) or an LTR with TAR deleted (Ad-HIVΔTARluc) (5) at twice-weekly intervals for 6 weeks. This system is highly responsive to Tat and allows



**FIG. 5. HIV-1 mRNA profile during long-term infection of MDM is the same irrespective of the time in culture before infection.** MDM from a single donor were infected with Ba-L at weekly intervals over 4 weeks, and then samples were taken weekly for a further 5 weeks after infection for analysis of virus production and mRNA abundance. (A) Kinetics of virus production as assessed by RT activity of culture supernatants from MDM infected 1 and 4 weeks after isolation, respectively, are shown. (B) Relative proportions of *tat*-1, *rev*-1, and *nef*-1 over time in cells infected 1 and 4 weeks after isolation. Expression of particular viral mRNA species in the same cells from which virus production was measured as shown in panel A were determined as a proportion of the total 1.8-kb mRNA by phosphorimaging analysis, and then this proportion was related to that present at peak expression at 21 days postinfection. wk, week.

one to determine Tat-specific HIV-1 LTR-mediated gene expression, which increases linearly with increasing levels of HIV-1 infection in a wide variety of cell types (5). In addition, the Ad-HIVΔTARluc recombinant virus controls for any effects of adenovirus superinfection that are not due to Tat. Forty-eight hours after addition of the recombinant adenoviruses, the cells were lysed and luciferase activity in the lysates was measured. Luciferase activity in HIV-infected MDM superinfected with Ad-HIVluc, a specific measure of Tat-dependent transactivation of the HIV-1 LTR (19), was very high, peaked 1 to 2 weeks after HIV infection (donor variable), and then declined dramatically over subsequent weeks to near background levels by 2 to 3 weeks (Fig. 6A). Virus production from the MDM cultures showed a pattern similar to that of Tat activity, except for the expected delay between peak Tat activity and maximal virus production (Fig. 6B). This delay was between 2 and 7 days, depending on the donor culture. In

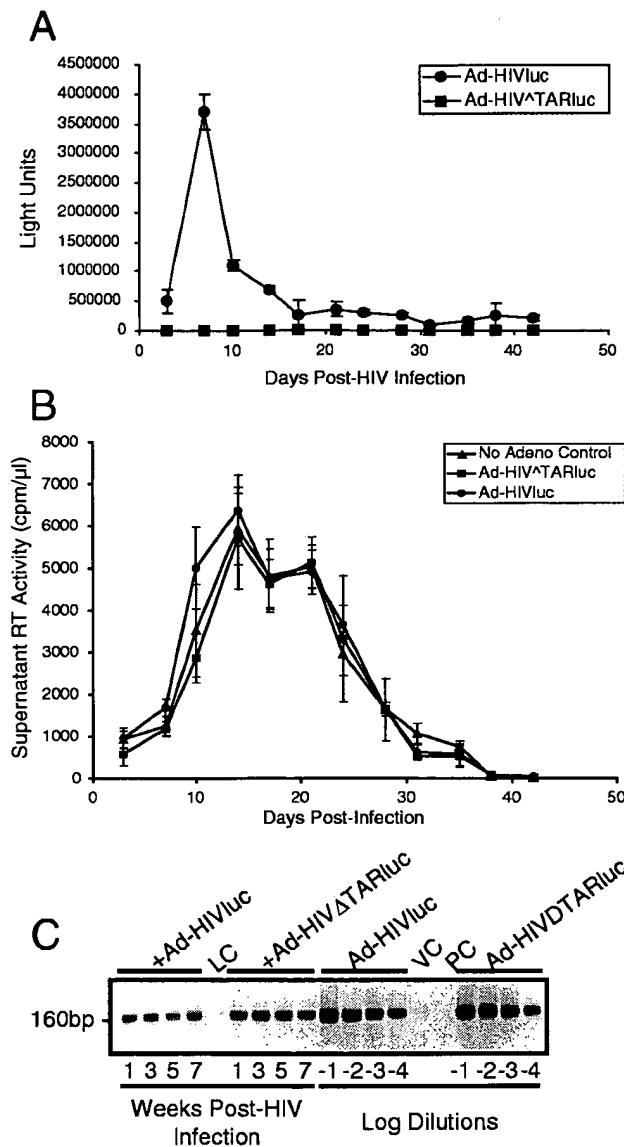


FIG. 6. Tat activity declines rapidly to near background levels during persistent HIV-1 infection in MDM. Tat activity during long-term HIV-1 infection of MDM was determined by superinfection with recombinant adenoviruses containing the wild-type HIV-1 LTR or the HIV-1 LTR with TAR deleted controlling the expression of a luciferase reporter gene (Ad-HIVluc and Ad-HIV $\Delta$ TARluc, respectively). (A) Luciferase activity was measured in cells lysed 48 h after superinfection and expressed as arbitrary light units. Lysates were diluted 10-fold to fall within the range measurable by the luminometer used. Background levels of cells not superinfected with adenovirus vectors were <8,000 light units. (B) Supernatant RT activity in the replicate MDM cultures from panel A superinfected with Ad-HIVluc or Ad-HIV $\Delta$ TARluc or maintained as nonsuperinfected controls (No Adeno Control). (C) PCR for luciferase DNA in 1-, 3-, 5-, and 7-week cell lysates from which luciferase activity was determined as described in panel A (left side) and in log dilutions of the standardized vector stocks (right side). LC, nonsuperinfected cell lysate control; VC, virus control; PC, PCR control. The examples shown are representative of the results obtained with samples from three donors.

contrast, the luciferase activity within the lysates from MDM infected with Ad-HIV $\Delta$ TARluc, which indicates the level of Tat-independent LTR-driven transcription in the cells, remained relatively constant throughout the course of the infection (Fig. 6A). This was not due to a lower superinfection efficiency of the Ad-HIV $\Delta$ TARluc compared to Ad-HIVluc virus, however, as the luciferase gene levels determined by PCR in the MDM lysates remained similar throughout (Fig. 6C). Replicate wells of HIV-infected MDM superinfected with either of the recombinant adenoviruses, or to which adenovirus was not added, produced essentially identical HIV growth curves (Fig. 6B).

Tat activity in infected PBMC cultures from three donors also increased over the first week of infection and then declined gradually over the next week as the cells became less viable and cell numbers decreased because of cytopathicity (Fig. 7A). This was mirrored by the RT activity in the culture supernatant (Fig. 7B), with Tat activity again preceding virus production by several days. Despite higher levels of HIV-1 replication in PBMC cultures (Fig. 6B and 7B), the peak luciferase activity in these cells was more than 1,000-fold lower than that found in MDM cultures (Fig. 6A and 7A), most likely because of the low expression level of the adenovirus receptor on lymphocytes (36).

**Exogenous Tat rescues virus production from long-term-infected MDM.** Given the low levels of *tat* mRNA and Tat protein activity in MDM from about 3 to 4 weeks after HIV infection, the decline in productive infection in these cells might result from suboptimal levels of Tat protein. When Tat protein was added to an MDM culture 2 to 3 weeks after the peak in virus expression at a concentration shown previously to induce HIV-1 production from the latently infected promonocytic cell line U1 (17), a spike in supernatant RT activity was found (Fig. 8A). RT activity in the culture supernatant began to rise 3 days after Tat addition, peaked at 10 days, and then began to decline again by 14 days (Fig. 8A). In the absence of exogenous Tat, RT activity declined from low to undetectable levels over the same 2-week period. This reactivation of virus production is likely to be in response to the provision of Tat to cells shown above to have very low Tat activity (Fig. 6A) and not result solely from any lipopolysaccharide (LPS) contaminating the protein preparation since we were unable to induce virus replication from long-term-infected macrophage cultures with LPS treatment (1 ng/ml), despite several attempts (unpublished data). Analysis of 1.8-kb mRNA within MDM again showed a decreased abundance of *tat* mRNA after the peak of infection. Following the addition of Tat protein, there was no change in the RNA profile from that found in control cells without exogenous Tat (Fig. 8B). Significantly, the levels of the *tat* mRNA species remained consistently low.

As found previously with the latently infected U1 and ACH2 cell lines (8, 22, 23), a similar but lower burst of virus production was induced by the addition of PMA or TNF- $\alpha$  to long-term-infected MDM (Fig. 8A). Activation by these factors occurs, at least in part, through translocation of the transcription factor NF- $\kappa$ B to the nucleus (27) and is independent of Tat.

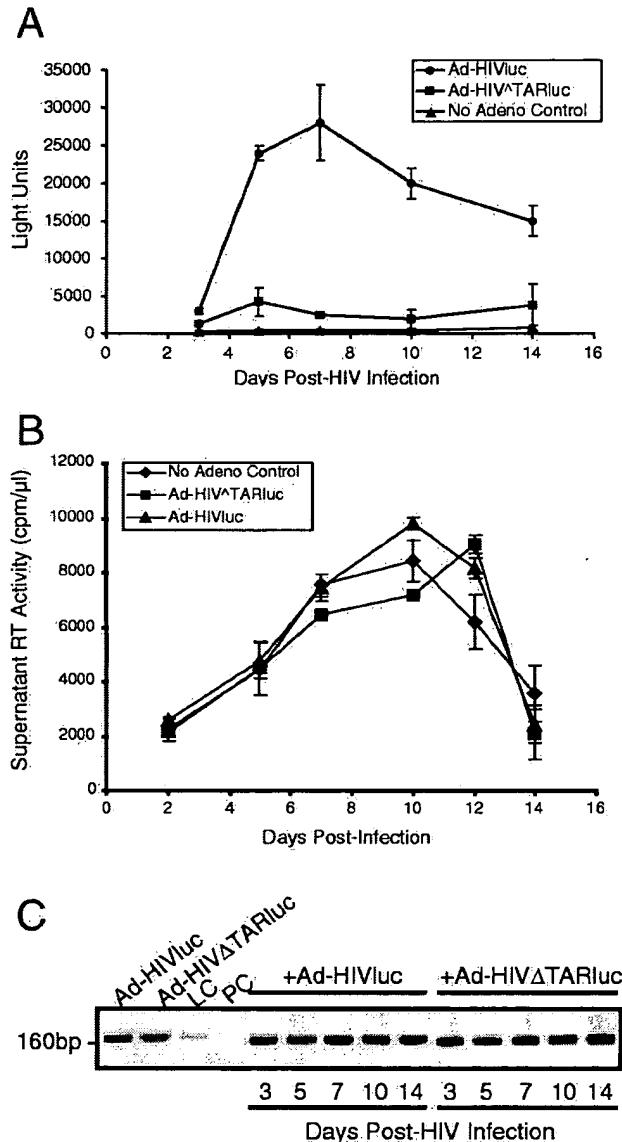


FIG. 7. Tat activity in HIV-1-infected PBMC. An analysis similar to that performed with MDM and shown in Fig. 6 was also done with PBMC cultures infected for 2 weeks. (A) Tat activity as determined by luciferase activity in superinfected cells (note that the lysates did not need to be diluted 1 in 10 as did the macrophages lysates in Fig. 6). (B) Virus production from the cultures. (C) Luciferase gene PCR in cell lysates and adenovirus stocks. The results shown are representative of the results obtained with samples from three donors. No Adeno Control, no adenovirus control; LC, nonsuperinfected cell lysate control; PC, PCR control.

## DISCUSSION

The genome length mRNA of HIV-1 contains many features, such as multiple alternative splice sites and exonic splice enhancers and ESS, that could modulate RNA splicing to change the outcome of viral infection. In this study, we examined the complex splicing profile of HIV-1 mRNA during infection of primary cells of the lymphocyte and macrophage lineages from the first hours of infection through to late times, when virus production wanes. Unlike earlier studies of the

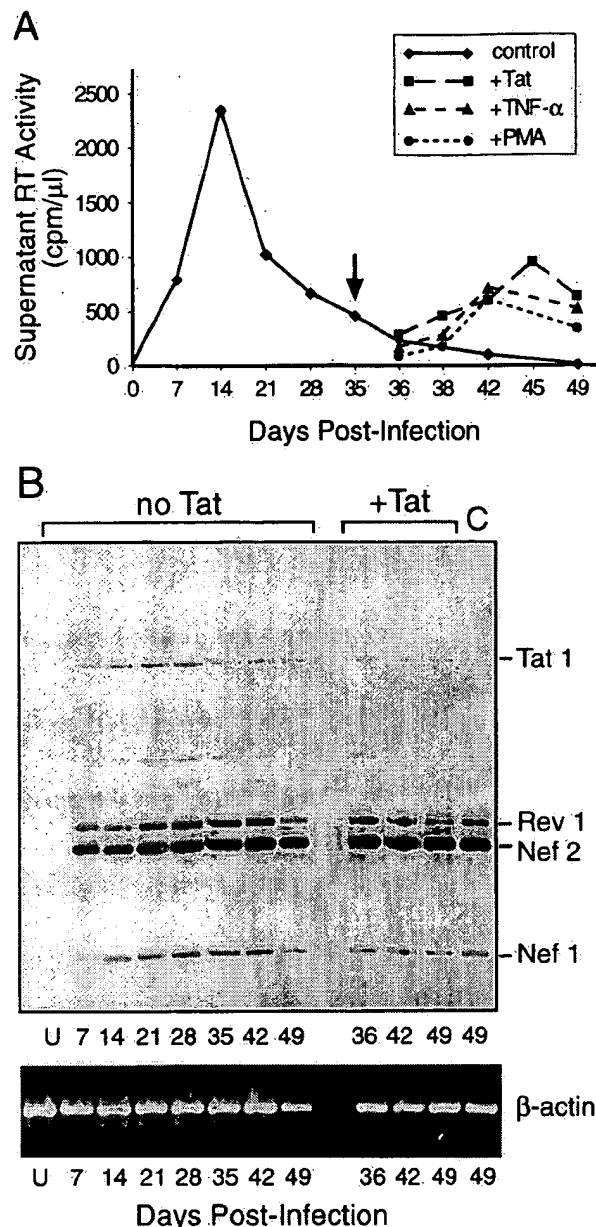


FIG. 8. A burst of HIV-1 production results from addition of exogenous Tat to persistently infected MDM. MDM from one donor were infected with Ba-L 7 days after isolation and maintained for 4 to 5 weeks before the addition of Tat protein (200 ng/well), TNF- $\alpha$  (100 ng/ml), or PMA (100 ng/ml) to the culture medium. The culture was then monitored for virion production and viral RNA expression for a further 2 weeks. (A) RT activity in supernatant from a representative culture infected for 5 weeks before any additions to the culture medium were made. The solid line represents the RT activity in supernatant from cells maintained in culture medium alone for 7 weeks following infection. Dotted lines represent the RT activity in the same cells after the addition of Tat, TNF- $\alpha$ , or PMA, respectively, on day 35 (arrow). (B) Viral 1.8-kb RNA (top) and  $\beta$ -actin mRNA (bottom) in cells from the culture shown in panel A. The left side of each panel represents mRNA from cells to which no Tat was added and that were lysed 7 to 49 days after infection. The right side represents RNAs from cells lysed 1, 7, and 14 days after addition of Tat (day 35 postinfection). U, RNA from uninfected cells; C, control infected cells to which protamine sulfate only was added.

kinetic accumulation of HIV-1 mRNAs (31, 32, 45), the reverse transcription-PCR method that we used could accurately measure the relative proportion of each of the alternative mRNA isoforms.

We found that HIV-1 1.8-kb mRNA accumulated 12 h earlier in lymphocytes than in MDM. This comparative delay in MDM agrees with the 12-h delay observed in one study of U937 cells (39) but not the 4-h delay reported in another study (32). It also agrees with our previous work in which we found that in fully susceptible MDM, compared to freshly isolated monocytes that are resistant to infection, reverse transcription and integration are detectable by 12 and 24 h postinfection, respectively (52). We determined that a fixed relative proportion of each of the 1.8-kb mRNAs appears simultaneously in MDM. Other studies that employed specific probes (32, 45) concluded that the *nef* mRNAs were the first expressed during infection; however, our results show that this interpretation stems from the relatively larger proportion of *nef* mRNA than the *rev* and *tat* RNAs. Primary lymphocytes and MDM produced the same array of spliced mRNAs; however, MDM had a lower relative abundance of the minor *tat*, *rev*, and *nef* isoforms that include noncoding exon 2 and/or 3. The most highly spliced *nef* mRNA, *nef-1*, was detected with lower abundance during infection of MDM than during infection of PBL and was not readily detected until late time points in a spreading infection of MDM. The requirement for Rev protein explains the delay in accumulation of the 4.0-kb series of HIV-1 mRNA compared to the 1.8-kb mRNAs (16, 20, 29, 34); however, a greater delay was evident in MDM than in PBL.

While there were the above-described relatively minor differences between lymphocytes and MDM during the first few weeks of infection, a much more significant difference was found in long-term-infected MDM just prior to and following the peak of viral infection in the macrophages, namely, a dramatic specific decrease in *tat* mRNAs greater than that found for any of the other spliced mRNAs. This specific decrease in *tat* was never observed in lymphocytes and was found to occur in macrophages as a consequence of infection rather than cell differentiation or maturation in culture. MDM are predominantly nondividing, and their expression of cellular mRNA, as indicated by the expression of the housekeeping gene  $\beta$ -actin, varies little, even over 8 weeks or more in culture, regardless of the HIV-1 status of the cells. Therefore, this specific decrease in *tat* mRNA was not due to a decline in cell numbers or viability but appears to coincide with the entry of MDM into a state of chronic or persistent infection with minimal virus production (at least at the level of sensitivity of a micro-RT assay). These persistently infected, long-term-cultured macrophages were still capable of producing virus, however, since addition of Tat protein was able to increase virus production from cells, reinforcing the potentially important role of low Tat levels in the long-term, nonproductive HIV-1 infection of MDM. Reactivation of virus production by Tat was achieved without altering the profile of viral RNA splicing from that seen at the time of Tat addition. This suggests that diminished splicing or stability of *tat*-specific mRNAs during long-term infection of MDM results in insufficient levels of Tat protein for efficient virion production, which is supported by the rapid decrease in Tat activity in these cells, determined with adenovirus vectors, after the first week or two of infection to near background

levels in MDM infected for a month or more. As expected, this rapid decrease was not found in infected lymphocyte cultures that continue to produce virus while the cells remain viable.

Several groups examining the chronically infected cell lines U1 and ACH2, which have been used extensively as models of HIV-1 latency (22, 23, 37, 43), have shown that mutations within *tat* and TAR, respectively, are responsible for the dormant infections seen within these cells (8, 17, 18). Analysis of *tat* cDNAs from U1 cells showed two distinct forms of mutated *tat*, each represented in one of two integrated DNA copies. One *tat* cDNA was shown to lack an AUG initiation codon, while the other had a point mutation (H13L) that caused a severe reduction in transcriptional elongation in Tat reporter assays (18). In a fashion analogous to this report of long-term-infected macrophages, the addition of Tat protein to the culture medium led to activation of HIV-1 production from U1 cells (8). In contrast, in the ACH-2 cell line, normal activity was not rescued by addition of Tat protein to the culture medium (8), showing that the point mutation in TAR in these cells was sufficient to explain their transcriptional dormancy (18).

CEM T cells chronically infected with HIV-1 gradually become latently infected over a 4- to 6-week period. The viral genes influencing the rate of shutdown in these cells were mapped, and the 3' region of the LTR was found to be the major determinant and the *tat/rev/vpu* region was found to contribute to a lesser extent (50). Since one of the five possible mutations in the LTR affecting HIV shutdown was in TAR, these findings also suggest that disruption of the Tat-TAR transcriptional axis can induce and maintain viral latency. In MDM, mutations in TAR would not explain our findings since the cultures remain responsive to exogenous Tat.

The diminished accumulation of Tat in long-term-infected MDM may result from altered RNA splicing, possibly induced by changes in cellular proteins that bind to the known ESS elements in the *tat* gene. Such events could reduce the expression of Tat sufficiently to severely disrupt Tat-TAR transcription and shut off virion production. The *tat* gene contains three ESS elements (ESS2, ESS2p, and ESS3) (2, 30, 57) that require the binding of hnRNPs of the A, B, or H group for their function of inhibiting splicing at adjacent upstream splice sites (9, 14, 30). These cellular factors and their role in HIV-1 splicing regulation have mostly been identified with HeLa cells. In addition, members of the serine/arginine-rich family of splicing factors have also been implicated in splicing regulation, usually by increasing splicing via exonic splice enhancer elements (59). Differential antagonism between these positive and negative regulators helps determine alternative exon usage (63). Although it is likely that such an interplay of these factors is also involved in alternative splicing regulation in macrophages, there is no direct evidence of this, nor whether this balance moves toward suppression of splicing during persistent infection in these long-lived cells.

The observed decline in *tat* mRNA and, subsequently, Tat activity to suboptimal levels for efficient virus production in macrophages could also be explained by the differential stability of *tat* mRNA relative to that of other HIV-1 transcripts during long-term infection of these cells. Various cellular genes are known to be regulated by controlling the rate at which their mRNAs decay, providing the cell with flexibility for

rapid changes in their expression. Several cytokine-, growth factor-, and proto-oncogene-encoding mRNAs, such as granulocyte-macrophage colony-stimulating factor, TNF- $\alpha$ , gamma interferon, interleukin-2, c-fos, c-myc, and c-jun, are regulated by differential RNA stability (reviewed in reference 61), while several viruses, such as herpesviruses and hepatitis C virus, also use this strategy to control the levels and kinetics of viral and cellular gene expression (21, 56). Whether this is a mechanism used by HIV-1 to regulate its replication in macrophages is not known.

Whatever mechanism is responsible for viral shutoff in macrophages, it appears to be induced in response to infection rather than as a consequence of differentiation or maturation of the cells per se. Irrespective of whether MDM are infected with HIV-1 after a week or so in culture, as is the common practice with this culture system, or maintained for a month or more before they are infected, the same pattern of *tat* mRNA decline several weeks after infection is seen. This might suggest that accumulation in these cells of a particular viral protein or proteins, perhaps Tat itself, leads to cellular changes that affect either the splicing regulation of *tat* mRNA or its relative stability. The resultant chronically, but nonproductively, infected macrophage may well be significant as a viral reservoir and an important means by which the virus can persist in the body.

Although we have been able to show that HIV-1 persists in peripheral blood monocytes *in vivo* (54), evidence of this pattern of chronic infection in monocytes or macrophages from tissue sites of HIV-infected individuals is lacking, most probably because of the difficulty in obtaining sufficient infected cells for such an analysis. We and others have, however, shown that low-level replication does continue in monocytes despite years of effective antiretroviral therapy (54, 64) and have speculated that this reflects continued local replication of HIV-1 in the tissue macrophage reservoir (12, 51), which is generally considered to be an important source from which virus can rapidly rebound (3, 10, 35). Our demonstration of this pattern of infection in the most commonly used *in vitro* model of HIV-1 infection in macrophages suggests that regulation of viral mRNA processing may prove to be an important mechanism by which macrophages become long-lived reservoirs of HIV-1, where dormant or low-level expression of virus assists in the evasion of immune clearance.

Determination of the mechanism of HIV-1 mRNA regulation in macrophages, whether it involves altered splicing or differential stability or another mechanism, may uncover novel therapeutic targets that can be used against these critical cells. Currently available drugs are, at best, poorly efficacious against chronically infected cells, and alternative treatments and approaches are required that will be active against all reservoirs of HIV-1, will restrict viral replication in and transmission from these cells, and will lead eventually to their elimination from the body. In the shorter term, the ability to control replication in macrophages should greatly assist with the problem of rapid rebound after cessation or interruption of even the best currently available regimens.

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## Stable and efficient gene transfer into the retina using an HIV-based lentiviral vector

HIROYUKI MIYOSHI\*, MASAYO TAKAHASHI\*†, FRED H. GAGE, AND INDER M. VERMA‡

Laboratory of Genetics, The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, CA 92037

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**ABSTRACT** The development of methods for efficient gene transfer to terminally differentiated retinal cells is important to study the function of the retina as well as for gene therapy of retinal diseases. We have developed a lentiviral vector system based on the HIV that can transduce terminally differentiated neurons of the brain *in vivo*. In this study, we have evaluated the ability of HIV vectors to transfer genes into retinal cells. An HIV vector containing a gene encoding the green fluorescent protein (GFP) was injected into the subretinal space of rat eyes. The GFP gene under the control of the cytomegalovirus promoter was efficiently expressed in both photoreceptor cells and retinal pigment epithelium. However, the use of the rhodopsin promoter resulted in expression predominantly in photoreceptor cells. Most successfully transduced eyes showed that photoreceptor cells in >80% of the area of whole retina expressed the GFP. The GFP expression persisted for at least 12 weeks with no apparent decrease. The efficient gene transfer into photoreceptor cells by HIV vectors will be useful for gene therapy of retinal diseases such as retinitis pigmentosa.

Inherited retinal degenerations define a genetically and clinically heterogeneous group of eye diseases. Retinitis pigmentosa is the most common form of retinal degeneration, affecting  $\approx 1$  in 3,500 people. Symptoms include progressive loss of visual field, night blindness, and the deposition of pigment in the retina. Although increasing numbers of genes responsible for retinal degenerative diseases have recently been identified, the underlying mechanism of retinal degeneration is not well understood and there are no adequate therapies for these diseases at present. Therefore, an efficient method of gene transfer into the retina provides a useful tool for both the investigation and treatment of retinal degeneration.

Many techniques have been tested for *in vivo* gene transfer into the retina. Nonviral methods, such as liposomes, are inefficient and only achieve transient expression of the transgene (1, 2). The majority of cells in the mature retina, including photoreceptor cells, are terminally differentiated and are incapable of proliferation. In this regard, vectors derived from retroviruses such as Moloney murine leukemia virus (MLV) are not useful because they require proliferation of the target cells for integration and stable expression. Consequently, the use of retroviral vectors are limited to *ex vivo* gene transfer (3, 4). Several other viral vectors, including herpes simplex virus, adenovirus and adeno-associated virus vectors, have been developed for gene delivery to nondividing cells such as neurons. However, transduction of photoreceptor cells or other neuronal retinal cells by means of these viral vectors has been inefficient, and sustained long-term expression has not been observed (5–12). Recently, Flannery *et al.* (13) have reported that the use of a rhodopsin promoter in adenovirus

associated virus vectors allowed efficient photoreceptor-targeted gene expression. Successful gene transfer to photoreceptor cells is important for gene therapy of retinal degenerative diseases, because degeneration primarily affects photoreceptor cells and most of the identified genes causing retinal degeneration are expressed specifically in photoreceptor cells (14).

We have described previously (15) a lentiviral vector system based on the HIV that can transduce nondividing cells *in vitro* and *in vivo*. Subsequently, we have shown efficient gene transfer and sustained long-term expression of the transgene in terminally differentiated neurons of the adult rat brain with HIV vectors (16, 17). In the present study, we have evaluated the ability of an HIV vector, which contains the green fluorescent protein (GFP) reporter gene under the control of the cytomegalovirus (CMV) promoter, to transduce retinal cells of rats. In addition, an HIV vector with the rhodopsin promoter was designed to direct photoreceptor-specific expression of the reporter gene. We report here efficient transduction of retinal cells using HIV vectors.

## MATERIALS AND METHODS

**Plasmid Constructs and Preparation of Viral Vectors.** pEGFP-C1 B/S was constructed by inserting a 13-bp *Spe*I linker at the *Bgl*II site of pEGFP-C1 (Clontech) to introduce two in-frame stop codons just upstream of *Xba*I site. A humanized, red-shifted GFP gene fragment, driven by the CMV promoter, was obtained from pEGFP-C1 B/S by digesting with *Ase*I, blunt-ending with the Klenow fragment of DNA polymerase I, and digesting with *Xba*I. This fragment was subcloned into the *Eco*RV and *Xba*I sites of pBluescript KS(+) (Stratagene), resulting in pKS-CMV-GFP. The HIV vector with the CMV promoter, pHR'-CMV-GFP, was constructed by inserting the 1,340-bp *Bam*HI-*Xba*I fragment containing the CMV promoter and the GFP gene from pKS-CMV-GFP into the same sites of pHR' vector (15). The HIV vector with the rhodopsin promoter, pHR'-Rho-GFP, was constructed by replacing the CMV promoter fragment in pHR'-CMV-GFP with the bovine rhodopsin promoter fragment from -2174 rhodopsin-*lacZ* fusion construct (a kind gift from D. J. Zack, ref. 18). The HIV-based vectors were generated as described (16). The MLV-based vector, pCLNCG, was derived from a pCL vector (19) and contains the same CMV-GFP gene expression cassette as pHR'-CMV-GFP and additionally the neomycin resistance gene. Cloning details are available upon request. The MLV-based vector was generated by the same

Abbreviations: CMV, cytomegalovirus; MLV, Moloney murine leukemia virus; GFP, green fluorescent protein; RPE, retinal pigment epithelium.

\*H.M. and M.T. contributed equally to this paper.

†Present address: Department of Ophthalmology & Visual Sciences, Graduate School of Medicine, Kyoto University, Sakyo-ku, Kyoto 606, Japan.

‡To whom reprint requests should be addressed at: Laboratory of Genetics, The Salk Institute for Biological Studies, P.O. Box 85800, San Diego, CA 92186-5800. e-mail: verma@salk.edu.

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procedure as the HIV-based vectors except using 293 gp/bsr cells, that stably express MLV *gag* and *pol* under the control of the CMV promoter. The titer of pH'R'-CMV-GFP and pCLNCG vectors was determined by infection of 208F rat fibroblasts, seeded in six-well plates at  $1 \times 10^5$  cells per well the day before infection, with serial dilutions of concentrated vector stocks in the presence of 8  $\mu$ g/ml of Polybrene (Aldrich). After overnight incubation, cell culture medium was changed and the cells further incubated for 2 days, and the number of GFP-positive cells was scored by fluorescence microscopy and/or fluorescence-activated cell sorting analysis to quantify the titer. After concentration by ultracentrifugation, titers of  $3-5 \times 10^8$  infectious units/ml on 208F cells were usually obtained. The titer of pH'R'-Rho-GFP vector was normalized for HIV-1 p24 antigen level, that was determined by ELISA (DuPont).

**In Vivo Delivery of Vectors.** Adult Fischer 344 rats (age 4 weeks) or pups (age 2 to 5 days) were anesthetized by i.p. injection of ketamin/acepromazine/xylazine or chilling on ice for 5 min, respectively. For pups, the eyeball was exposed by an incision in the eyelid, parallel to the future edge of the open eyelid. Subretinal injections were performed under an operating microscope. A small incision was made in sclera, and 0.5–1.5  $\mu$ l of vector suspension ( $\approx 5 \times 10^5$  infectious units) was injected through the incision into the subretinal space using a glass capillary connected with a 5- $\mu$ l syringe (Hamilton). For adult rats, a total of eight eyes were injected with the pH'R'-CMV-GFP vector, eight with the pH'R'-Rho-GFP vector, 10 with the pCLNCG vector, and 10 with saline. For pups, a total of 44 eyes were injected with the pH'R'-CMV-GFP, 24 with the pH'R'-Rho-GFP, and 44 with the pCLNCG.

**Detection of GFP Expression.** Rats were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer at 2, 6, or 12 weeks after injection. Eyes were enucleated and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 6–8 h at 4°C followed by cryoprotection by soaking in 30%

sucrose at 4°C overnight. Eyes were frozen in O.C.T. compound (Tissue-Tek) on dry ice and 20- $\mu$ m-thick cryostat sections were cut parallel to the vertical meridian of the eye. GFP expression were analyzed by confocal laser scanning microscopy (Bio-Rad). The images were generated electronically with Adobe PHOTOSHOP. Fluorescent signals were collected under the same conditions and no modification on intensity was made, so that fluorescent intensity of each section can be compared. Cell nuclei were counterstained with 4',6'-diamino-2-phenylindole and observed with a UV filter. The same fields were examined with  $\times 40$  objective lens equipped with grid to determine the number of GFP-positive cells and the total number of photoreceptor cells. At least 2,000 cells in four separate fields of the most efficiently transduced eye in each group were scored.

## RESULTS

To study gene transfer and expression *in vivo*, we used a humanized, red-shifted GFP gene as a reporter under the control of the CMV promoter or the rhodopsin promoter in an HIV-based vector system (15). Since the virus particles are pseudotyped with the vesicular stomatitis virus G glycoprotein, the vector can be concentrated to high titer and transduce a broad range of tissues. The titer of HIV vector with the CMV promoter was determined by infecting dividing 208F rat fibroblast cells. However, the titer of HIV vector with the rhodopsin promoter was difficult to determine, because rhodopsin promoter activity is extremely low in established nonretinal cell lines and even in Y79 retinoblastoma cells. Therefore, equal amounts of recombinant viruses normalized for HIV-1 p24 antigen level were used. For comparison, vesicular stomatitis virus G glycoprotein pseudotyped MLV-based vector with the CMV promoter, matched for transducing activity on 208F cells, was used. Approximately  $5 \times 10^5$  infectious units of vector suspension were injected into the subretinal space of

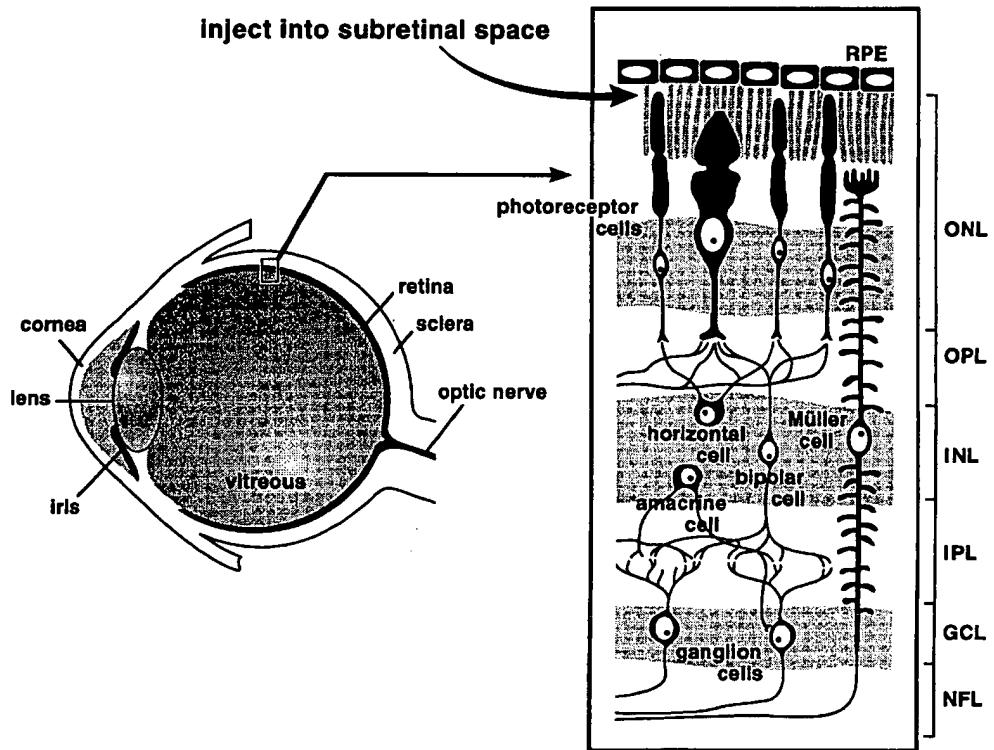


FIG. 1. Schematic diagram of the eye and retina. The site of subretinal injection is indicated. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; NFL, nerve fiber layer.

both eyes of 2- to 5-day-old pups or 4-week-old adult rats (Fig. 1). At serial time points 2 to 12 weeks postvector delivery, rats were sacrificed and eye tissue was cryosectioned and analyzed for GFP expression by fluorescence microscopy.

Fig. 2 shows retinal sections from pups 2 and 12 weeks postinjection. In eyes injected with the HIV vector with the CMV promoter, the GFP gene was intensely expressed in a very high proportion of RPE cells as well as in a large number of photoreceptor cells (Fig. 2 *a* and *d*). In addition, a few GFP-positive cells were unambiguously identified as bipolar cells (see arrowhead in Fig. 2*d*) on the basis of morphological characteristics, and no positive cells were seen in other types of retinal cells. No green fluorescence was detected in the eyes of control animals that received only saline (data not shown). In contrast, the MLV vector with the CMV promoter transduced RPE and photoreceptor cells inefficiently (Fig. 2 *c* and *f*). Additionally, small numbers of positive cells were found in the neural retina, including bipolar cells and mostly Müller cells (see Fig. 2*c*), around the injection site. For the MLV vector, the intensity of the green fluorescence in photoreceptor cells was much less than that transduced with HIV vectors. Injection of the HIV vector with the rhodopsin promoter resulted in GFP expression only in photoreceptor cells (Fig. 2 *b* and *e*). Note that unlike the expression in nonretinal cells *in vitro*, GFP expression with the rhodopsin promoter in photoreceptor cells was more efficient than that with the CMV promoter. Although the number of GFP-positive cells varied highly in each eye and even in the different sections from the same eye, most successfully transduced eyes showed that photoreceptor cells in >80% of the whole area of the retina viewed through multiple sections expressed GFP (representative example is shown in Fig. 3). The pattern and the total number of GFP-positive cells did not change appreciably for at least 12 weeks after injection of HIV vectors or MLV vector (Fig. 2 *a*–*f*). Several inflammatory cells such as macrophages were observed in the eyes surrounding the injection site at 2 weeks after injection of vectors. This inflammation was likely due to the surgical damage during injection, because a similar

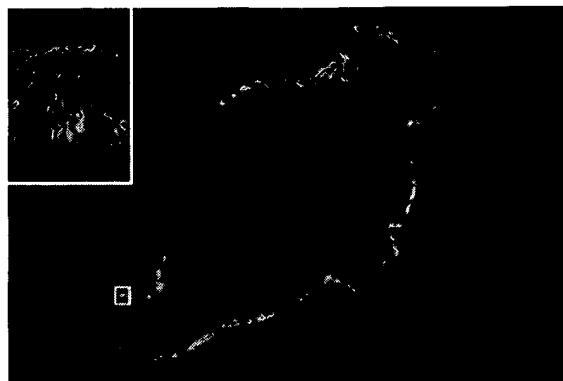


FIG. 3. Extent of GFP expression in the retina. Transverse section of the pup eye 2 weeks after injection of the HIV vector with the rhodopsin promoter. Note that photoreceptor cells in whole area of retinal section express GFP. Original magnification,  $\times 40$ . (Inset) Higher magnification of the area indicated by  $\square$ .

phenomenon was observed in control animals that had saline injections (data not shown). No inflammation was observed at 6 and 12 weeks postinjection. To compare the transduction efficiency of photoreceptor cells, sections of eyes were stained with 4',6' diamino-2-phenylindole and were then examined by fluorescence microscopy to determine the percentage of GFP-positive photoreceptor cells (Fig. 4). The difference between HIV and MLV vectors with the CMV promoter is greater than 5-fold ( $\approx 16\%$  vs. 3% for HIV and MLV vectors, respectively). The HIV vector with the rhodopsin promoter transduced about 2.5 times more photoreceptor cells than the HIV vector with the CMV promoter. The increased efficiency may be due to high levels of expression of the transgene driven by the photoreceptor-specific rhodopsin promoter, because equal amounts of HIV vectors were injected per eye.

We next evaluated the ability of HIV vectors to transduce cells in the adult rat eye. Fig. 5 shows that the pattern of GFP

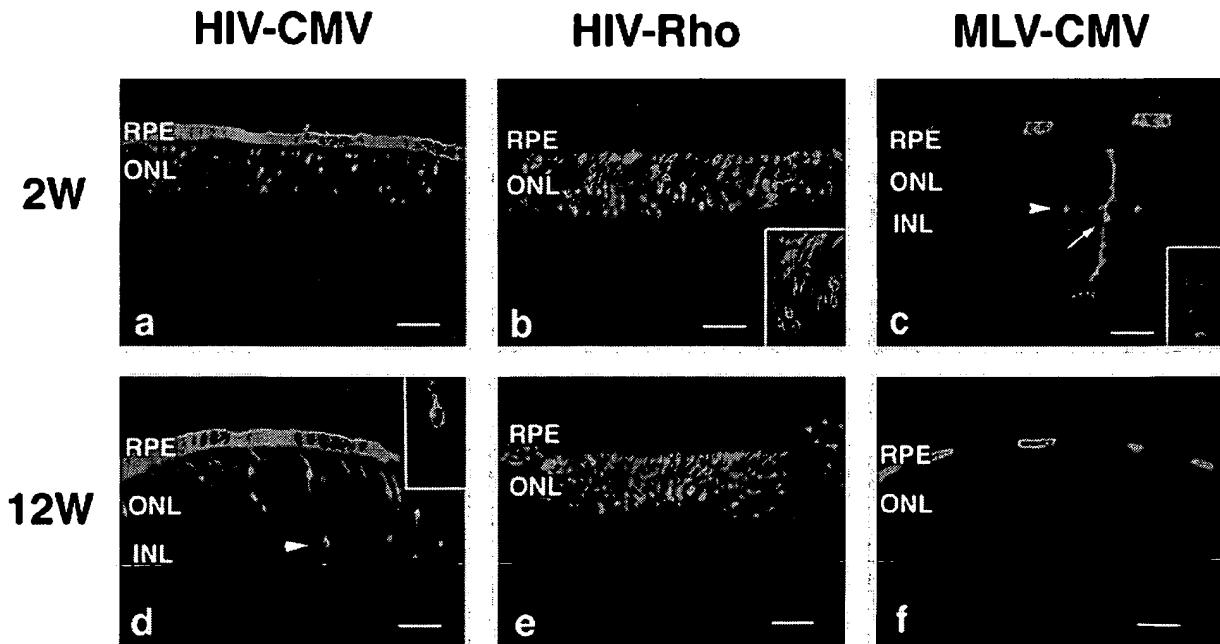


FIG. 2. Expression of GFP in the retina of rat pups 2 and 12 weeks after injection. (*a* and *d*) HIV vector with the CMV promoter. Arrowhead in *d* indicates GFP-positive bipolar cell. (*Inset*) Higher magnification of GFP-positive bipolar cell. (*b* and *e*) HIV vector with the rhodopsin promoter. Note that only photoreceptor cells are GFP positive. (*Inset*) Higher magnification of GFP-positive photoreceptor cells. (*c* and *f*) MLV vector with the CMV promoter. Arrowhead in *c* indicates GFP-positive bipolar cell. Arrow in *c* indicates GFP-positive Müller cell. (*Inset*) Higher magnification of GFP-positive bipolar cell. Original magnification,  $\times 400$ . Bars = 50  $\mu\text{m}$ .

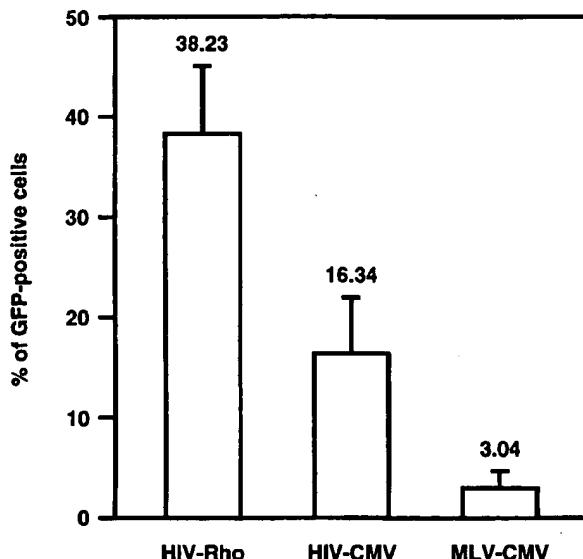


FIG. 4. Proportion of GFP-positive photoreceptor cells in the outer nuclear layer 2 weeks after injection into the eyes of pups. HIV-Rho, HIV vector with the rhodopsin promoter; HIV-CMV, HIV vector with the CMV promoter; MLV-CMV, MLV vector with the CMV promoter. At least 2,000 cells in 4 separate fields of the most efficiently transduced eye in each group were scored. Data represent means  $\pm$  SD. *P* values are calculated by Student's *t* test. *P* = 0.0180, HIV-Rho vs. HIV-CMV. *P* = 0.0008, HIV-Rho versus MLV-CMV. *P* = 0.0141, HIV-CMV vs. MLV-CMV.

expression in adult rat eyes injected with HIV vectors is similar to that of pups but the transduction efficiency is decreased. With the CMV promoter, the distribution of GFP-positive cells in RPE was more restricted than that in pups, and only photoreceptor cells around the injection site expressed GFP (Fig. 5 *a* and *d*). With the rhodopsin promoter, GFP-positive

cells were also seen only in the outer nuclear layer, where photoreceptors are located, surrounding the injection site (Fig. 5 *b* and *e*). There were no significant differences in the pattern and the percentage of GFP-positive cells between 2 and 12 weeks postinjection of HIV vectors. In contrast, the MLV vector transduced only a few RPE cells, and no GFP-positive cells were found in the neural retina, presumably because the majority of the retinal cells are terminally differentiated in adult eyes (Fig. 5*c*). Little or no expression of GFP was detected at 12 weeks postinjection (Fig. 5*f*).

## DISCUSSION

The present study demonstrates that HIV-based vectors can efficiently transfer and express a transgene in retinal cells, especially in RPE and photoreceptor cells. In adult rats, expression of the transgene was restricted around the injection site. One possible explanation for this is that the interstitial space between photoreceptor cells is more tight in adults than in pups, and HIV vector particles may not diffuse into the interphotoreceptor and subretinal space. To transduce a larger number of retinal cells in the adult, multiple subretinal injections are needed. An alternative way of delivery is intravitreous injection with high numbers of vector particles.

Expression of the transgene persisted for at least 12 weeks with no apparent decrease. Since outer segments of photoreceptor cells continuously turn over by phagocytosis of RPE cells, long-term expression of the transgene in photoreceptor cells is presumably due to stable integration of the transgene into the genome of cells with self-renewing properties. This idea is supported by our previous study (16) using an HIV vector with mutant integrase, in which we found that expression of the transgene in rat brain depends on its ability to integrate. Recently, we have also demonstrated long-term expression of the transgene in liver and muscle using HIV vectors (T. Kafri, U. Blömer, F.H.G., and I.M.V., unpublished work). Therefore, long-term expression in the retina as well as

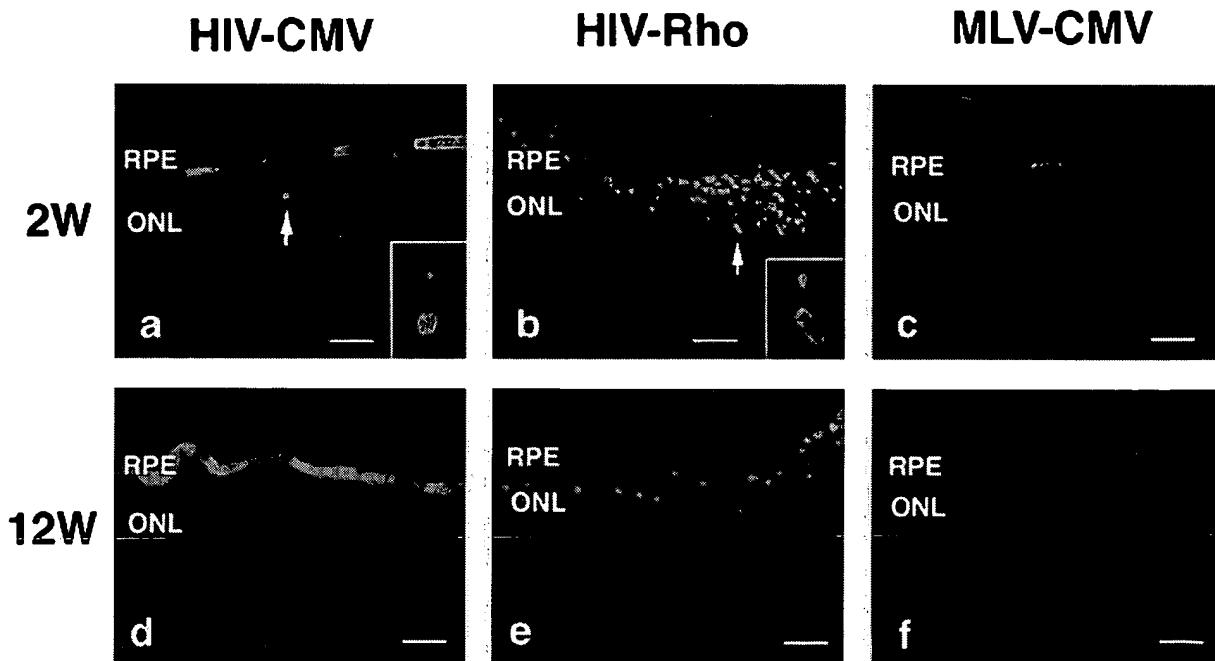


FIG. 5. Expression of GFP in the adult retina 2 and 12 weeks after injection. (*a* and *d*) HIV vector with the CMV promoter. (*b* and *e*) HIV vector with the rhodopsin promoter. (*c* and *f*) MLV vector with the CMV promoter. Arrows in *a* and *b* indicate GFP-positive photoreceptor cells. Insets in *a* and *b*, higher magnification of GFP-positive photoreceptor cells. Original magnification,  $\times 400$ . Bars = 50  $\mu$ m.

the brain cannot be explained by the relative immune privilege in these tissues.

MLV requires proliferation of target cells for integration and expression of the transgene (20). Hence the ability of MLV vectors to infect neuronal retinal cells in pups is likely due to a small proportion of retinal cells capable of proliferation and differentiation at the time of injection. In fact, autoradiographic studies with  $^3\text{H}$ -thymidine have shown that 20–30% of retinal cells still proliferate in the early postnatal period (21). In addition, lineage analysis using MLV vectors to mark clones has shown the existence of proliferating multipotential progenitor cells in postnatal rodent retina (22).

Several other virus-based vectors, including those of herpes simplex virus, adenovirus and adeno-associated virus, have been tested for gene delivery to retinal cells. The transduction frequency of these vectors to photoreceptor cells was extremely low and expression of transgenes was transient or diminished over long terms (5–12). However, these vectors can transduce RPE cells with relatively high efficiency (5–9, 11, 12). Our studies here also show efficient and sustained transduction in RPE cells with HIV and MLV vectors. RPE cells may efficiently take up viral particles by their intrinsic phagocytic activity.

It was of interest to evaluate the specificity and efficiency of transgene expression using a cell-type specific promoter in the HIV-based vector delivery system. We chose the rhodopsin promoter as a cell-type specific promoter to drive the expression of the reporter gene, because rhodopsin is the most abundant photoreceptor-specific protein ( $4 \times 10^7$  rhodopsins per rod) (23). Additionally, it has been shown in transgenic mouse experiments that the DNA fragment of the rhodopsin promoter we used in this study can direct photoreceptor-specific expression (18). The use of a rhodopsin promoter in the HIV vector led not only to photoreceptor-specific expression but also to higher expression levels than with the CMV promoter. Recently, Flannery *et al.* (13) have also reported that adeno-associated virus vectors with the rhodopsin promoter can achieve efficient transduction of photoreceptor cells.

*In vivo* gene delivery to photoreceptor cells is important for gene therapy of inherited retinal degenerative diseases such as retinitis pigmentosa, because most of the identified genes responsible for retinal degeneration so far are expressed specifically in photoreceptor cells (14). Our results suggest that the HIV vector is a promising vehicle for delivering therapeutic genes to photoreceptor cells. This can be tested in mouse models of retinitis pigmentosa, including the spontaneous mutants *rd* and *rds* (24–26), and mice carrying a targeted disruption of the rhodopsin gene or the  $\gamma$  subunit of rod cGMP phosphodiesterase gene (27, 28). Transgenic experiments in *rd* and *rds* mice have shown that introduction of wild-type genes, the  $\beta$  subunit of rod cGMP phosphodiesterase and peripherin genes, respectively, can rescue retinal degeneration (29, 30). However, it is not yet clear when and how many photoreceptor cells have to be rescued from degeneration to maintain minimal visual function. Hence, the HIV-based vector delivery system offers substantial promise for both the investigation and treatment of retinal diseases.

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## *cis*-Acting Sequences Involved in Human Immunodeficiency Virus Type 1 RNA Packaging

JANE F. KAYE, JENNIFER H. RICHARDSON,† AND ANDREW M. L. LEVER\*

Department of Medicine, Addenbrooke's Hospital,  
Cambridge CB2 2QQ, United Kingdom

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We have previously described a series of human immunodeficiency virus type 1-based vectors in which efficient RNA encapsidation appeared to correlate with the presence of a 1.1-kb *env* gene fragment encompassing the Rev-responsive element (RRE). In this report, we explore in detail the role of the RRE and flanking *env* sequences in vector expression and RNA encapsidation. The analysis of a new series of vectors containing deletions within the *env* fragment failed to identify a discrete packaging signal, although the loss of certain sequences reduced packaging efficiency three- to fourfold. Complete removal of the *env* fragment resulted in a 100-fold decrease in the vector transduction titer but did not abolish RNA encapsidation. We conclude that the RRE and 3' *env* sequences are not essential for human immunodeficiency virus type 1 vector encapsidation but may be important in vectors in which a heterologous gene has been placed adjacent to the 5' packaging signal, potentially disrupting its structure.

Retroviral RNA packaging is a specific process involving interactions among *cis*-acting sequences in the RNA genome and viral structural proteins. In human immunodeficiency virus type 1 (HIV-1), an important packaging signal has been located in the 5' untranslated region (UTR) downstream of the major splice donor, the presence of which is necessary for genomic RNA encapsidation (1, 3, 8). Differing conclusions as to whether this region is sufficient to direct the encapsidation of HIV-1-based vectors containing heterologous genes have been reached. Several groups using a transient COS-1 cell packaging system have reported the successful encapsidation of HIV-1-based vectors which contain only the long terminal repeats (LTRs), the 5' UTR, and in some cases part of the *gag* gene (2, 6, 11, 12, 17). Using replication-competent helper virus to package HIV-1-based vectors stably expressed in CD4<sup>+</sup> T-cell lines, we were unable to demonstrate the packaging of this type of vector, although vectors which contained a 1.1-kb *env* fragment in addition were packaged with high efficiency (13). This system has the advantages of simulating expression of stably integrated provirus and rigorously excluding any possibility of DNA-mediated gene transfer, which is a problem associated with the high levels of plasmid DNA present in transient transfection systems. In addition, packaging of retroviral RNA varies significantly among different cell lines (unpublished observations).

To further define the role of the Rev-responsive element (RRE) and flanking sequences in vector RNA expression and encapsidation, we constructed a series of vectors containing deletions in the 3' *env* sequence and studied the effects of these on RNA encapsidation by a wild-type helper virus. All vectors were derived from HXBc2, an infectious proviral clone of the human T-cell leukemia virus (HTLV) IIIB isolate (4), and contain a simian virus 40 origin of replication. Restriction sites, where given, refer to positions in the HXBc2 genome (Los

Alamos database numbering, in which position 1 is the first base of the 5' LTR). The vectors are illustrated in Fig. 1. The construction of LRPL has previously been described (13). Briefly, it contains a puromycin acetyltransferase gene (*puro*) (10) inserted at a position analogous to that of the *nef* gene, between an introduced *NotI* site (8740) (20) and an *Xba*I site (8897). The *puro* gene is expressed from the 5' LTR as a spliced transcript. The sequences between *Clal* (830) and *Bgl*II (7621), encoding *gag*, *pol*, and the 5' part of *env*, have been deleted. The second *tat* and *rev* exons and the RRE are retained. A series of deletions based on LRPL were constructed as follows. *Xba*I and *Sal*I sites were introduced at either end of the RRE, at positions 7704 and 8063, respectively, by oligonucleotide-directed mutagenesis (7). The sequences of the mutagenic oligonucleotides were 5' GGGTGCTACTCTAGAG GTTCAATTTCAC 3' (*Xba*I) and 5' CCAGAGATTATTA GTCGAQCTAGCATTCC 3' (*Sal*I). The introduction of each mutation was confirmed by restriction analysis and DNA sequencing. Deletions spanning the 1.1-kb *env* gene fragment in LRPL were constructed by removing sequences between various restriction sites (Fig. 1). Vector plasmids containing the *puro* selectable marker were transfected into the CD4<sup>+</sup> T-cell line Jurkat-*tat* (14) by electroporation. Stable cell lines were selected by the addition of puromycin (0.5 µg/ml) to the culture medium.

The vector transduction and RNA encapsidation levels of the vectors shown in Fig. 1 were determined following infection of the vector lines with the HIV-1 isolate HTLV IIIB. The progeny virus was harvested 7 days later and filtered through a 0.45-µm-pore-size membrane. Vector transduction titers and RNA encapsidation levels were determined as follows. For vector titration on HeLa T4 cells (9), 100-mm-diameter petri dishes seeded with 5 × 10<sup>5</sup> cells were infected with dilutions of virus. Puromycin selection (0.75 µg/ml) was applied 24 h after infection. Puromycin-resistant colonies were counted 10 days later, after the cells were fixed in 4% formal saline and stained with 0.1% toluidine blue. To quantitate the levels of vector RNA packaging, the amounts of vector RNA and genomic helper virus RNA in virion particles from HTLV IIIB-infected vector lines were compared. Virion RNA was extracted and

\* Corresponding author. Mailing address: Department of Medicine, Addenbrooke's Hospital, Hills Rd., Cambridge CB2 2QQ, United Kingdom. Phone: 1223 336849. Fax: 1223 336846.

† Present address: Division of Human Retrovirology, Dana-Farber Cancer Institute, Boston, MA 02115.

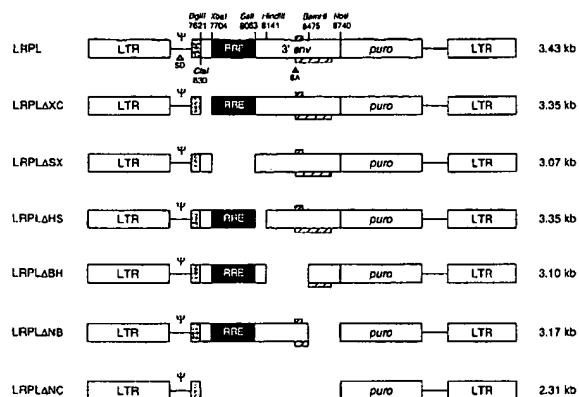


FIG. 1. HIV-1 vectors. All vectors contain the 5' and 3' LTRs, the 5' UTR, and the first 43 bp of *gag*. The stippled boxes show *gag* sequences. The major splice donor (SD) and the *tat* and *rev* splice acceptors (SA) are marked. The RRE is indicated. Hatched boxes indicate the second exons of *tat* and *rev*.

normalized for reverse transcriptase activity, and duplicate slot blots were prepared and hybridized with vector-specific (*puro*) or helper virus-specific (*pol*) probes as previously described (13). Bound probe was measured with an Instant Imager (Packard), which measures radioactivity in real time. To overcome differences in the lengths and specific activities of the two probes, a reference sample in which the stoichiometry of *puro*-to-*pol*-hybridizing sequences is 1:1 was included. The reference sample consisted of RNA from a puromycin-resistant HIV-1 isolate containing the *puro* gene in place of *nef*. The vector encapsidation level, expressed as a percentage of the wild-type helper virus level, was calculated with the following formula:

$$\frac{\text{PURO cpm of sample}}{\text{POL cpm of sample}} / \frac{\text{PURO cpm of reference}}{\text{POL cpm of reference}} \times 100$$

The results are shown in Table 1. All of the vectors were able to transduce target cells expressing the CD4 molecule; however, the efficiency of LRPLANC was reduced 100-fold. The levels of vector RNA encapsidation (Fig. 2 and Table 1) were comparable to those of the parental vector, LRPL, with the exception of LRPLANC. Possible reasons for the low level of LRPLANC encapsidation are discussed below. Vectors based on LRPL containing either the introduced *Xba*I or *Sall* restriction site, or both, were able to transduce CD4<sup>+</sup> target cells with titers comparable to that of the parental vector (data not shown). The packaging efficiency of some vectors was reduced up to fourfold; however, none of the *env* sequences deleted

TABLE 1. Vector transduction titers and encapsidation levels

Vector	HeLa T4 (CFU/ml) <sup>a</sup>	Encapsidation level (%) <sup>b</sup>
LRPL	$4.47 \times 10^3$	6.8
LRPLAXC	$1.33 \times 10^3$	3.1
LRPLABH	$3.04 \times 10^3$	1.8
LRPLAHS	$1.05 \times 10^4$	12.0
LRPLASX	$1.06 \times 10^3$	3.1
LRPLANB	$3.37 \times 10^3$	3.6
LRPLANC	$1.30 \times 10^1$	1.6

<sup>a</sup> Normalized for a reverse transcriptase activity of  $10^4$  cpm/ml. The results shown are means of three or more experiments.

<sup>b</sup> Relative to the level of wild-type virus.

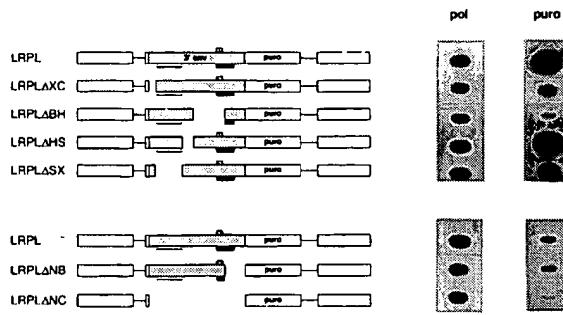


FIG. 2. Vector RNA encapsidation. Virus produced from HTLV IIIB-infected vector lines was harvested, and the virion RNA was hybridized with *puro* or *pol* gene probes.

from LRPL appeared to be essential for vector RNA encapsidation.

While quantitation of vector RNA packaging as described above indicates whether the vectors are packageable, such data do not provide information regarding the relative packaging efficiencies of different RNAs. These were determined by estimating the level of full-length (FL) vector RNA in the cells and comparing this with the amount subsequently detected in the virions by slot blot analysis. To compare the relative amounts of FL vector RNA, Northern (RNA) blots of total RNA from HTLV IIIB-infected vector lines were prepared and hybridized with a *puro* gene probe as described previously

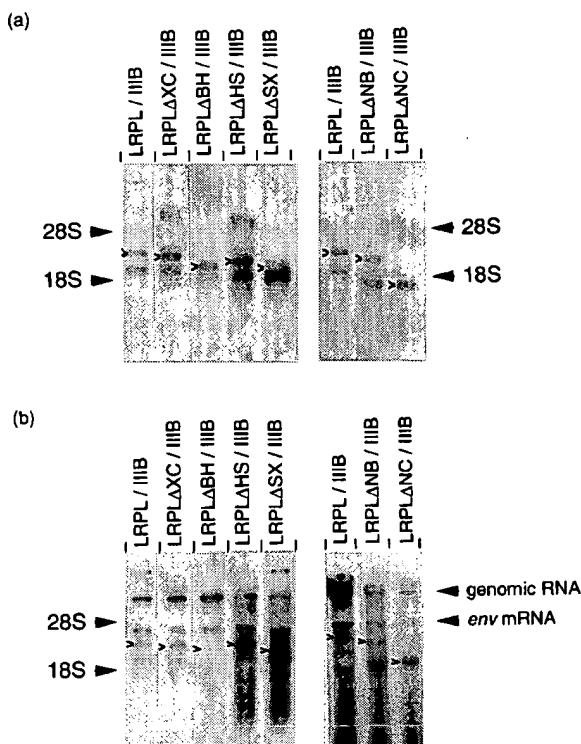


FIG. 3. Comparison of vector RNA expression levels. Total RNA from HTLV IIIB-infected vector lines was hybridized with a *puro* gene probe (a) or an LTR probe (b). Carets indicate the positions of FL vector RNA. The positions of 18S and 28S rRNAs are marked. The positions of genomic RNA and *env* mRNA are indicated.

TABLE 2. Vector expression and packaging efficiencies

Vector	Relative abundance of FL vector <sup>a</sup>	Encapsulation level <sup>b</sup>	Packaging efficiency
HTLV IIIB	100	100	1.0
LRPL	59.2	6.8	0.12
LRPL $\Delta$ XC	41.7	3.1	0.07
LRPL $\Delta$ BH	16.2	1.8	0.11
LRPL $\Delta$ HS	81.4	12.0	0.15
LRPL $\Delta$ SX	ND <sup>c</sup>	3.1	ND
LRPL $\Delta$ NB	110	3.6	0.03
LRPL $\Delta$ NC	141	1.6	0.01

<sup>a</sup> Expressed as a percentage of the amount of FL helper virus RNA in the infected vector lines.

<sup>b</sup> Expressed as a percentage of the amount of helper virus RNA present in the virions.

<sup>c</sup> ND, not determined.

(13) (Fig. 3a). Hybridization with an LTR probe allowed the relative levels of vector and HTLV IIIB genomic RNA to be directly compared (Fig. 3b). The packaging efficiency of the wild-type genome was assigned a value of 1.0, and the packaging efficiencies of FL vector RNAs were calculated with the following formula:

$$\frac{\text{amount of FL vector packaged}}{\text{amount of HTLV IIIB genome packaged}} / \frac{\text{amount of FL vector in the cell}}{\text{amount of HTLV IIIB genome in the cell}}$$

The results are shown in Table 2. The packaging efficiencies of the vectors containing deletions in the 1.1-kb *env* gene sequence are comparable to that of the parental vector LRPL. The variation in the RNA encapsidation levels (shown in Fig. 2 and Table 1) appears to be due to variations in the level of FL vector RNA expressed in the helper virus-infected cells (Fig. 3). Deletion of the RRE in the vector LRPL $\Delta$ SX and other vectors lacking the RRE (data not shown) did not result in a reduction in vector RNA expression and encapsidation, indicating that in the absence of *cis*-repressive sequences (15), the RRE is not absolutely required for efficient expression and encapsidation of FL vector RNA, although the lack of the RRE may have led to increased splicing. The relative abundance of FL LRPL $\Delta$ SX was not determined, as it was not possible to distinguish between FL and spliced vector RNAs by the method used. The vector in which the entire *env* gene has been deleted, LRPL $\Delta$ NC, was transduced at very low levels and packaged with 10-fold lower efficiency than that of the parent vector, despite the relatively high level of FL vector RNA available for packaging. While it is not essential for RNA encapsidation, the *env* fragment does appear to contribute to efficient RNA packaging, and part of its effect may be due to the sequences acting as a spacer between the heterologous gene and the 5' packaging signal.

The role of the 5' UTR in directing encapsidation of HIV-1 vectors based on LRPL was examined by a quantitative RNase protection assay. Transient cotransfection of COS-1 cells with vector and helper virus constructs was used to compare encapsidation of LRPL and a construct lacking the 5' UTR, pSVIIIfenv3-2. This plasmid has been previously described (18, 19). It contains the HIV-1 *rev* and *env* genes (nucleotides [nt] 5496 to 8897) under the control of 5' LTR sequences (nt 288 to 535, corresponding to -167 to +80 with respect to the RNA start site), and the 3' LTR is replaced by simian virus 40 polyadenylation sequences. A *gag-pol-env* expressor was used as a helper virus for the cotransfection assay. pBCCX-CSF (a kind gift from Alan Cann) contains HIV-1 JR-CSF sequences 640 to 8915 encoding the *gag*, *pol*, and *env* genes; the 5' and 3'

LTRs have been replaced by human cytomegalovirus immediate early promoter and polyadenylation sequences, respectively. COS-1 cells were transiently transfected with vector and helper virus constructs by a DEAE-dextran transfection protocol (16). Cells and supernatants were harvested 48 h later. Cytoplasmic RNA was prepared by a standard protocol (5). For RNA extraction from virions, particles were purified from tissue culture supernatant through 20% sucrose cushions as previously described (13). Virus particles were lysed in proteinase K buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 10 mM EDTA, 1% sodium dodecyl sulfate, 100 µg of proteinase K per ml, and 100 µg of tRNA per ml) for 30 min at 37°C. After two phenol-chloroform extractions and one chloroform extraction, the RNA was precipitated with ethanol and stored at -70°C.

A DNA template, KSII $\psi$ CS, containing *Scal* (313)-to-*Clai* (830) sequences of HXBc2 inserted into the *EcoRV* and *Clai* sites in the polylinker of Bluescript KSII (Stratagene) was used for synthesis of radiolabelled RNA probes. KSII $\psi$ CS was linearized with *Xba*I, and <sup>32</sup>P-labelled antisense riboprobes were synthesized with T3 RNA polymerase by using an *in vitro* transcription kit (Promega). Reagents for RNase protection assays were obtained from a commercially available kit (Ambion, Austin, Tex.). Cytoplasmic RNA or RNA extracted from pelleted particles, normalized for reverse transcriptase activity, was analyzed by RNase protection assay according to the manufacturer's recommended protocol. For size determination, <sup>32</sup>P-labelled RNA markers, synthesized with an RNA Century Marker template set (Ambion), were run in parallel. The predicted sizes of the protected fragments are shown in Fig. 4.

The results of a typical RNase protection assay are shown in Fig. 5. The input probe is of the expected size (Fig. 5, lane 8), and no signal is detected with the control tRNA (lane 7). Helper virus (pBCCX-CSF) bands are of the predicted sizes (Nar1-SD, 100 nt, and Nar1-gag, 170 nt). A fragment corresponding to FL LRPL vector RNA (375 nt) was detected in cytoplasmic RNA (Fig. 5, lane 2). Spliced RNA (289 nt) and RNA corresponding to the 3' LTR (238 nt) were also detected (Fig. 5, lane 2). FL vector RNA was detected in particles released from cells transfected with LRPL and helper virus constructs (Fig. 5, lane 5). The level of FL vector RNA compared with that of spliced RNA was enriched in the particles by comparison with the levels present in the cells, indicating the

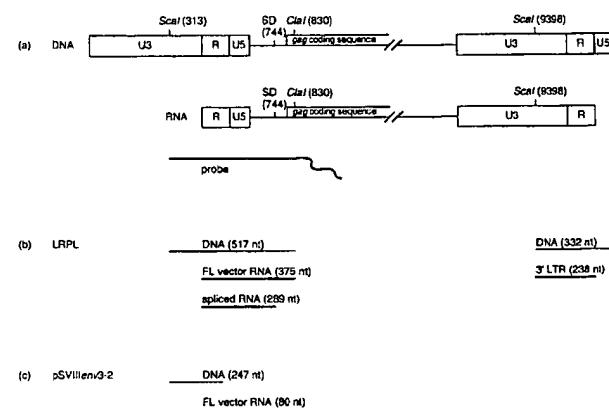


FIG. 4. Predicted sizes of protected fragments for the RNase protection assay. (a) KSII $\psi$ CS riboprobe is complementary to HIV-1 nt 313 to 830. (b) The predicted sizes of protected fragments for vector LRPL. (c) The predicted sizes of protected fragments for vector pSVIIIfenv3-2.

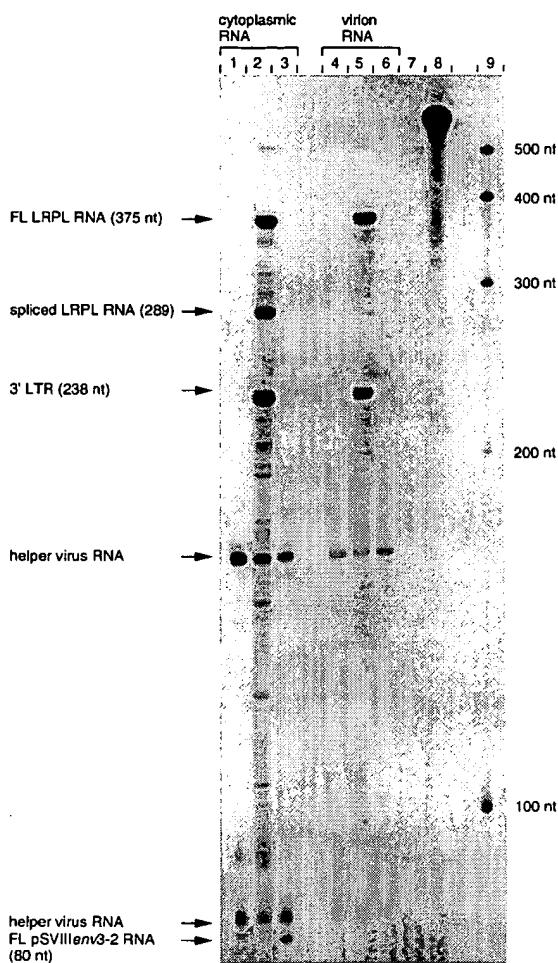


FIG. 5. Packaging of RNA into virions released from transfected COS-1 cells. Shown is an autoradiogram of gel-separated fragments of  $^{32}$ P-labelled riboprobe KSII $\psi$ CS resulting from RNase protection with cytoplasmic RNA (lanes 1 to 3) or virion RNA (lanes 4 to 6) prepared from COS-1 cells cotransfected with LRPL and pBCCX-CSF (lanes 2 and 5), pSVIIIenv3-2 and pBCCX-CSF (lanes 3 and 6), or pBCCX-CSF alone (lanes 1 and 4). Protection with tRNA alone is shown in lane 7, and riboprobe without RNase treatment is shown in lane 8.  $^{32}$ P-labelled RNA size markers are shown in lane 9. Fragment sizes are indicated at the left side of the panel. The positions of the protected helper virus fragments are indicated.

specificity of encapsidation of RNAs containing the 5'  $\psi$  sequence. A fragment corresponding to the FL pSVIIIenv3-2 vector RNA (80 nt) was detected in cytoplasmic RNA (Fig. 5, lane 3). This fragment was not detected in particles released from cells cotransfected with pSVIIIenv3-2 and helper virus constructs (Fig. 5, lane 6). This result confirms the importance of the previously identified packaging signal in the 5' UTR for encapsidation of HIV-1 RNA. The failure of the *env* gene sequences in pSVIIIenv3-2 to direct encapsidation of the vector RNA further indicates that *env* sequences are not sufficient to direct encapsidation of HIV-1 RNA.

From previously published work, it was unclear whether the different requirements for packaging vectors in COS cells and T cells reflected cell-specific phenomena or differences between transient and stable vector expression or were related to aspects of vector construction. In this report, we address the latter by characterization of the role of *env* gene sequences in

vector RNA expression and encapsidation. Analysis of a series of vectors based on LRPL demonstrated no absolute requirement for the 3' *env* region for encapsidation, although deletion of the entire region significantly inhibited packaging. That this was not completely abolished is shown by comparison with the negative control pSVIIIenv3-2, which, despite containing the entire *env* gene and being expressed at adequate levels in the cytoplasm, is completely nonpackageable. This also confirms the essential nature of the 5' UTR in encapsidation. We previously reported that when a heterologous gene is placed near the 5' UTR packaging signal, encapsidation is reduced or abolished (13). The findings reported here are in agreement with this. Point mutation of the *gag* ATG also appears to be detrimental. Our previous work demonstrated that some of these very poorly packageable vectors may be rendered packageable by inclusion of 3' *env* sequences downstream of the heterologous gene. In this paper, we show that this effect is not due to the presence of an essential *cis*-acting packaging signal in *env*, as *env* sequences can be omitted entirely from a packageable vector construct. However, the same region can enhance vector packaging when placed upstream of a heterologous gene. The practical implications of these findings are that it would appear important to include viral sequences between the 5' packaging signal and any heterologous gene in vectors based on HIV-1. In addition, vector RNA packaging may be enhanced by inclusion of the 3' *env* region in a vector construct. Studies such as these will aid in the development of HIV-based vectors, which to date have shown relatively low infectious titers, and also in the development of packaging cell lines based on lentiviruses.

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## A Third-Generation Lentivirus Vector with a Conditional Packaging System

TOM DULL,<sup>1</sup> ROMAIN ZUFFEREY,<sup>2</sup> MICHAEL KELLY,<sup>1</sup> R. J. MANDEL,<sup>1</sup>  
MINH NGUYEN,<sup>1</sup> DIDIER TRONO,<sup>2</sup> AND LUIGI NALDINI<sup>1\*</sup>

*Cell Genesys, Foster City, California,<sup>1</sup> and Department of Genetics and Microbiology,  
University of Geneva Medical School, Geneva, Switzerland<sup>2</sup>*

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Vectors derived from human immunodeficiency virus (HIV) are highly efficient vehicles for in vivo gene delivery. However, their biosafety is of major concern. Here we exploit the complexity of the HIV genome to provide lentivirus vectors with novel biosafety features. In addition to the structural genes, HIV contains two regulatory genes, *tat* and *rev*, that are essential for HIV replication, and four accessory genes that encode critical virulence factors. We previously reported that the HIV type 1 accessory open reading frames are dispensable for efficient gene transduction by a lentivirus vector. We now demonstrate that the requirement for the *tat* gene can be offset by placing constitutive promoters upstream of the vector transcript. Vectors generated from constructs containing such a chimeric long terminal repeat (LTR) transduced neurons in vivo at very high efficiency, whether or not they were produced in the presence of Tat. When the *rev* gene was also deleted from the packaging construct, expression of *gag* and *pol* was strictly dependent on Rev complementation in *trans*. By the combined use of a separate nonoverlapping Rev expression plasmid and a 5' LTR chimeric transfer construct, we achieved optimal yields of vector of high transducing efficiency (up to  $10^7$  transducing units [TU]/ml and  $10^4$  TU/ng of p24). This third-generation lentivirus vector uses only a fractional set of HIV genes: *gag*, *pol*, and *rev*. Moreover, the HIV-derived constructs, and any recombinant between them, are contingent on upstream elements and *trans* complementation for expression and thus are nonfunctional outside of the vector producer cells. This split-genome, conditional packaging system is based on existing viral sequences and acts as a built-in device against the generation of productive recombinants. While the actual biosafety of the vector will ultimately be proven in vivo, the improved design presented here should facilitate testing of lentivirus vectors.

Lentiviruses have attracted the attention of gene therapy investigators (45) for their ability to integrate into nondividing cells (8, 15, 16, 25, 26). We previously developed replication-defective vectors from the lentivirus human immunodeficiency virus (HIV) and showed that they transduce target cells independent of mitosis (32). The vectors proved highly efficient for in vivo gene delivery and achieved stable long-term expression of the transgene in several target tissues, such as the brain (5, 33), the retina (31), and the liver and muscle of adult rats (21). A major concern, however, is the biosafety of vectors derived from a highly pathogenic human virus.

The complexity of the lentivirus genome may be exploited to build novel biosafety features in the design of a retrovirus vector. In addition to the structural *gag*, *pol*, and *env* genes common to all retroviruses, HIV contains two regulatory genes, *tat* and *rev*, essential for viral replication, and four accessory genes, *vif*, *vpr*, *vpu*, and *nef*, that are not crucial for viral growth in vitro but are critical for in vivo replication and pathogenesis (27).

The Tat and Rev proteins regulate the levels of HIV gene expression at transcriptional and posttranscriptional levels, respectively. Due to the weak basal transcriptional activity of the HIV long terminal repeat (LTR), expression of the provirus initially results in small amounts of multiply spliced transcripts coding for the Tat, Rev, and Nef proteins. Tat increases dramatically HIV transcription by binding to a stem-loop structure (transactivation response element [TAR]) in the nascent RNA,

thereby recruiting a cyclin-kinase complex that stimulates transcriptional elongation by the polymerase II complex (46). Once Rev reaches a threshold concentration, it promotes the cytoplasmic accumulation of unspliced and singly spliced viral transcripts, leading to the production of the late viral proteins. Rev accomplishes this effect by serving as a connector between an RNA motif (the Rev-responsive element [RRE]), found in the envelope coding region of the HIV transcript, and components of the cell nuclear export machinery. Only in the presence of Tat and Rev are the HIV structural genes expressed and new viral particles produced (27).

In a first generation of HIV-derived vectors (32), viral particles were generated by expressing the HIV type 1 (HIV-1) core proteins, enzymes, and accessory factors from heterologous transcriptional signals and the envelope of another virus, most often the G protein of the vesicular stomatitis virus (VSV G) (9) from a separate plasmid. In a second version of the system, the HIV-derived packaging component was reduced to the *gag*, *pol*, *tat*, and *rev* genes of HIV-1 (51). In either case, the vector itself carried the HIV-derived *cis*-acting sequences necessary for transcription, encapsidation, reverse transcription, and integration (2, 4, 22, 24, 29, 30, 32, 35). It thus encompassed, from the 5' to 3' end, the HIV 5' LTR, the leader sequence and the 5' splice donor site, approximately 360 bp of the *gag* gene (with the *gag* reading frame closed by a synthetic stop codon), 700 bp of the *env* gene containing the RRE and a splice acceptor site, an internal promoter (typically the immediate-early enhancer/promoter of human cytomegalovirus [CMV] or that of the phosphoglycerokinase gene [PGK]), the transgene, and the HIV 3' LTR. Vector particles are produced by cotransfection of the three constructs in 293T cells (32). In this design, significant levels of transcription from the vector

\* Corresponding author. Mailing address: Cell Genesys, 342 Lakeside Dr., Foster City, CA 94404. Phone: (650) 425-4474. Fax: (650) 358-8636. E-mail: luigin@cellgenesys.com.

LTR and of accumulation of unspliced genomic RNA occur only in the presence of Tat and Rev.

Here, we demonstrate that the *trans*-acting function of Tat becomes dispensable if part of the upstream LTR in the transfer vector construct is replaced by constitutively active promoter sequences. Furthermore, we show that the expression of *rev* in *trans* allows the production of high-titer HIV-derived vector stocks from a packaging construct which contains only *gag* and *pol*. This design makes the expression of the packaging functions conditional on complementation available only in producer cells. The resulting gene delivery system, which conserves only three of the nine genes of HIV-1 and relies on four separate transcriptional units for the production of transducing particles, offers significant advantages for its predicted biosafety.

#### MATERIALS AND METHODS

**Transfer vector constructs.** pHR'CMV-LacZ and pHR'CMV-Luciferase have been described elsewhere (32). pHR2 is a lentivirus transfer vector in which the polylinker and downstream *nef* sequences up to the *Kpn*I site of pHR' have been replaced with a *Clal*/*Spel*/*SnaBI*/*Sma*I/*Bam*H/*Sac*II/*Eco*RI polylinker. pHR2 was generated by replacing the 3.7-kb *Clal*-*Sac*I fragment of pHR'CMVlacZ with a 607-bp *Clal*-*Sac*I fragment generated by PCR using pHR'CMVlacZ as the template with oligonucleotide primers 5'-CCATCGATGGACTAGTCTACGTA TCCCCGGGGACGGGATCCCGGAATTCCGTTAACGACCAATGAC-3' and 5'-TTATAATGTCAAAGCCCTCTC-3', followed by digestion with *Clal* and *Sac*I.

pHR2PGK-NGFR, pHR2CMV-NGFR, and pHR2MFG-NGFR are lentivirus transfer vectors in which the truncated low-affinity nerve growth factor receptor (NGFR) (6) transgenes under the control of the murine PGK, human CMV, and Moloney leukemia virus (MLV) promoters, respectively, have been inserted into the polylinker of pHR2. The pHR2PGK-NGFR transgene encodes no intron sequences, the pHR2CMV-NGFR vector includes the intron from plasmid pMD (34), and the pHR2MFG-NGFR vector contains the MLV intron from MFG-S (34).

pRRL, pRLL, pCCL, and pCLL are lentivirus transfer vectors containing chimeric Rous sarcoma virus (RSV)-HIV or CMV-HIV 5' LTRs and vector backbones in which the simian virus 40 polyadenylation and (enhancerless) origin of replication sequences have been included downstream of the HIV 3' LTR, replacing most of the human sequence remaining from the HIV integration site. In pRRL, the enhancer and promoter (nucleotides -233 to -1 relative to the transcriptional start site; GenBank accession no. J02342) from the U3 region of RSV are joined to the R region of the HIV-1 LTR. In pRLL, the RSV enhancer (nucleotides -233 to -50) sequences are joined to the promoter region (from position -78 relative to the transcriptional start site) of HIV-1. In pCCL, the enhancer and promoter (nucleotides -673 to -1 relative to the transcriptional start site; GenBank accession no. K03104) of CMV were joined to the R region of HIV-1. In pCLL, the CMV enhancer (nucleotides -673 to -220) was joined to the promoter region (position -78) of HIV-1. Exact sequences and details of construction are available on request.

pHR2hPGK-GFP, pCCLhPGK-GFP, pCLLhPGK-GFP, pRRLhPGK-GFP, and pRLLhPGK-GFP are lentivirus transfer vectors containing the enhanced green fluorescent protein (eGFP) (750-bp *Bam*H/*Not*I fragment from pEGFP-1; Clontech) coding region, under the control of the human PGK promoter (nucleotides 5 to 516; GenBank accession no. M11958), inserted into the polylinker region of each parental vector. pRRLhPGK was obtained by deletion of the *Xba*I-*Bam*H fragment containing the PGK promoter from pRRLhPGK-GFP.

pRRLhPGK.GFP.SIN-18 is a vector in which 3' LTR sequences from -418 to -18 relative to the U3/R border have been deleted from pRLLhPGK.GFP (52).

**Packaging constructs.** The *tat*-defective packaging construct pCMVΔR8.93 was obtained by swapping an *Eco*RI-*Sac*I fragment from plasmid R7/pneo(-) (12) with the corresponding fragment of pCMVΔR8.91, a previously described plasmid expressing Gag, Pol, Tat, and Rev (51). This fragment has a deletion affecting the initiation codon of the *tat* gene and a frameshift created by the insertion of an *Mlu*I linker into the *Bsu*3I site as described previously. pCMVΔR8.74 is a derivative of pCMVΔR8.91 in which a 133-bp *Sac*I fragment, containing a splice donor site, has been deleted from the CMV-derived region upstream of the HIV sequences to optimize expression.

pMDLg/p is a CMV-driven expression plasmid that contains only the *gag* and *pol* coding sequences from HIV-1. First, *pkat2Lg/p* was constructed by ligating a 4.2-kb *Clal*-*Eco*RI fragment from pCMVΔR8.74 with a 3.3-kb *Eco*RI-*Hind*III fragment from *pkat2* (14) and a 0.9-kb *Hind*III-*Not*I fragment from *pkat2* along with an *Not*I-*Clal* linker consisting of synthetic oligonucleotides 5'-CATGGGT GCGAGAGCGTCAGTATTAAGCGGGGAGAATTAGAT-3' and 5'-CG ATCTAATTCTCCCCGCTTAACTGACGCTCTCGCACC-3'. Next, pMDLg/p was constructed by inserting the 4.25-kb *Eco*RI fragment from *pkat2Lg/p* into the *Eco*RI site of pMD-2. pMD-2 is a derivative of pMD.G (34) in which the pXF3 plasmid backbone of pMD.G has been replaced with a

minimal pUC plasmid backbone and the 1.6-kb VSV G-encoding *Eco*RI fragment has been removed.

pMDLg/pRRE differs from pMDLg/p by the addition of a 374-bp RRE-containing sequence from HIV-1 (HXB2) immediately downstream of the *pol* coding sequences. To generate pMDLg/pRRE, the 374-bp *Not*I-*Hind*III RRE-containing fragment from pHR3 was ligated into the 9.3-kb *Not*I-*Bgl*II fragment of pVL1393 (Invitrogen) along with a *Hind*III-*Bgl*II oligonucleotide linker consisting of synthetic oligonucleotides 5'-AGCTTCGGCGGA-3' and 5'-GATCTCC GCGGA-3' to generate pVL1393RRE (pHR3 was derived from pHR2 by the removal of HIV *env* coding sequences upstream of the RRE sequences in pHR2). A *Not*I site remains at the junction between the *gag* and RRE sequences. pMDLg/pRRE was then constructed by ligating the 380-bp *Eco*RI-*Sst*II fragment from pVL1393RRE with the 3.15-kb *Sst*II-*Nde*I fragment from pMD-2FIX (pMD-2FIX is a human factor IX-containing variant of pMD-2 which has an *Sst*II site at the 3' end of the factor IX insert), the 2.25-kb *Nde*I-*Avr*I fragment from pMDLg/p, and the 3.09-kb *Avr*II-*Eco*RI fragment from pkat1Lg/p (14).

pRSV-Rev and pTK-Rev (generous gifts of T. Hope, Salk Institute) are *rev* cDNA-expressing plasmids in which the joined second and third exons of HIV-1 *rev* are under the transcriptional control of the RSV U3 and herpes simplex virus type 1 thymidine kinase (TK) promoters, respectively. Both expression plasmids utilize polyadenylation signal sequences from the HIV LTR in a pUC118 plasmid backbone.

**Vector production and assays.** Vectors were produced by transient transfection into 293T cells as previously described (33), with the following modifications. A total of  $5 \times 10^6$  293T cells were seeded in 10-cm-diameter dishes 24 h prior to transfection in Iscove modified Dulbecco culture medium (JRH Biosciences) with 10% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100  $\mu$ g/ml) in a 5% CO<sub>2</sub> incubator, and the culture medium was changed 2 h prior to transfection. A total of 20  $\mu$ g of plasmid DNA was used for the transfection of one dish: 3.5  $\mu$ g of the envelope plasmid pMD.G, 6.5  $\mu$ g of packaging plasmid, and 10  $\mu$ g of transfer vector plasmid. The precipitate was formed by adding the plasmids to a final volume of 450  $\mu$ l of 0.1  $\times$  TE (1  $\times$  TE is 10 mM Tris [pH 8.0] plus 1 mM EDTA) and 50  $\mu$ l of 2.5 M CaCl<sub>2</sub>, mixing well, then adding dropwise 500  $\mu$ l of 2 $\times$  HEPES-buffered saline (281 mM NaCl, 100 mM HEPES, 1.5 mM Na<sub>2</sub>HPO<sub>4</sub> [pH 7.12]) while vortexing and immediately adding the precipitate to the cultures. The medium (10 ml) was replaced after 14 to 16 h; the conditioned medium was collected after another 24 h, cleared by low-speed centrifugation, and filtered through 0.22- $\mu$ m-pore-size cellulose acetate filters. For *in vitro* experiments, serial dilutions of freshly harvested conditioned medium were used to infect 10<sup>5</sup> cells in a six-well plate in the presence of Polybrene (8  $\mu$ g/ml). Viral p24 antigen concentration was determined by immunocapture (Alliance; DuPont-NEN). Vector batches were tested for the absence of replication-competent virus by monitoring p24 antigen expression in the culture medium of transduced SupT1 lymphocytes for 3 weeks. In all cases tested, p24 was undetectable (detection limit, 3 pg/ml) once the input antigen had been eliminated from the culture. Transducing activity was expressed in transducing units (TU).

**Northern blot analysis.** Total RNA was isolated from 1  $\times$  10<sup>7</sup> to 2  $\times$  10<sup>7</sup> cells harvested at confluence by using RNASol B as suggested by the manufacturer; 10 to 20  $\mu$ g of RNA was loaded per well on 1% agarose gels, using NorthernMax (Ambion, Austin, Tex.) reagents as described by the manufacturer. Transfer was to Zetabind membranes (Cuno Inc., Meriden, Conn.) by either capillary transfer or pressure blotting (Stratagene). <sup>32</sup>P-labeled probes were made by random priming.

**Intracerebral injection of vectors.** Twelve Fischer 344 male rats weighing approximately 220 g were obtained from Harlan Sprague-Dawley (Indianapolis, Ind.). The rats were housed with access to ad libitum food and water on a 12-h light/dark cycle and were maintained and treated in accordance with published National Institutes of Health guidelines. All surgical procedures were performed with the rats under isoflurane gas anesthesia, using aseptic procedures. After a rat was anesthetized in a sleep box, it was placed in a small animal stereotaxic device (Kopf Instruments, Tujunga, Calif.) using the earbars, which do not break the tympanic membrane. The rats were randomly divided into one control and four treatment groups. After the rats were placed in the stereotaxic frame, 2  $\mu$ l of lentivirus vector concentrated by ultracentrifugation at 50,000  $\times$  g for 140 min at 20°C (33) in phosphate-buffered saline (PBS) was injected consecutively into the striatum in both hemispheres over 4 min at a rate of 0.5  $\mu$ l/min (coordinates, AP 0.0, LAT  $\pm$ 3.0, DV -5.5, -4.5, -3.5 with the incisor bar set at 3.3 mm below the intra-aural line [36]), using a continuous-infusion system as described previously in detail (28). During the injection, the needle was slowly raised 1 mm in the dorsal direction every 40 s (3-mm total withdrawal). One minute after cessation of the injection, the needle was retracted an additional 1 mm and then left in place for an additional 4 min before being slowly withdrawn from the brain.

**Histology.** One month after vector injection, each animal was deeply anesthetized with intraperitoneal pentobarbital and perfused through the aorta with sterile PBS, followed by ice-cold 4% paraformaldehyde perfusion. The brains were removed from the skulls, postfixed in 4% paraformaldehyde by immersion for 24 h, and then transferred into a 30% sucrose-PBS solution for 3 to 4 days, until the brains sank to the bottom of their containers. The brains were then frozen on dry ice, and 40- $\mu$ m-thick coronal sections were cut on a sliding microtome. Sections were collected in series in microtiter well plates that contained a glycerin-based antifreeze solution, and they were kept at -30°C until further processing. Immunocytochemistry was performed according to the general pro-

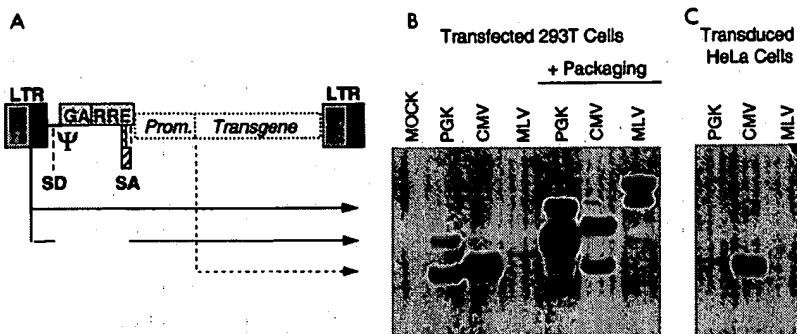


FIG. 1. Northern analysis of the RNA expression from lentivirus vectors. Three pH2R vectors carrying an expression cassette for the same transgene (truncated low-affinity NGFR) and driven by three different promoters (PGK, CMV, and retroviral MFG) were analyzed in producer and transduced cells. Total RNA was extracted and analyzed by Northern blotting with a probe specific for the transgene sequence. (A) Schematic of the vector construct depicts the species of RNA driven by the internal promoter (Prom.; broken arrow, shorter transcript) and the viral LTR (solid arrows, longer transcripts; the two species differ for the splicing of the viral intron). The splice donor and acceptor sites (SD and SA), the packaging sequence ( $\Psi$ ), the truncated gag sequence (GA), and the RRE are indicated. (B) The vector constructs were transfected in 293T cells without or with the packaging construct. (C) Vector particles produced by the 293T transfectants were used to transduce HeLa cells. In the absence of the viral transactivators, supplied by the core packaging construct only in the producer cells, vector expression occurs mainly from the internal promoter. Note the dramatic enhancement of the upstream transcription and the accumulation of unspliced RNA (carrying the  $\Psi$  sequence) in the presence of the packaging construct. In the transduced cells, the LTR is silenced. Note that the three expression cassettes differ in the size of the promoters and 5' untranslated sequence. In each case, the smallest RNA species represents transcripts initiated from the internal promoter, while the intermediate-size and larger species correspond to spliced and unspliced LTR-driven RNAs, respectively.

cedure described previously (44). After several PBS rinses and an incubation in 3% hydrogen peroxide, the sections were placed in a 3% normal goat serum. The sections were then incubated in the primary anti-GFP antibody (1:1,000; Clontech, Palo Alto, Calif.) in 1% normal goat serum–0.1% Triton X-100 overnight at room temperature. After rinsing, the sections were incubated in the biotinylated rabbit anti-goat secondary antibody (Vector, Burlingame, Calif.) for 3 h. After rinsing, the sections were incubated with horseradish peroxidase-streptavidin and then reacted by using a purple chromagen kit (VIP; Vector), mounted, dried, dehydrated, and coverslipped.

## RESULTS

**Tat is required to produce a vector of efficient transducing activity.** To investigate the role of Tat in the production of transducing particles, expression from lentivirus vectors was first examined by Northern analysis (Fig. 1). The patterns of RNAs induced by transfer vectors in which the transgene was driven by an internal PGK, CMV, or retrovirus MFG promoter were studied in both producer and target cells. In transfected 293T cells, expression occurred mainly from the internal promoter. When a packaging construct expressing both Tat and Rev was cotransfected, a dramatic enhancement of transcription from the LTR was observed, with an accumulation of unspliced vector RNA. In cells transduced with the vectors, that is, in the absence of Tat and Rev, transcription from the LTR was almost completely suppressed, the residual transcripts underwent splicing, and the internal promoter was responsible for most of the expression.

A packaging plasmid carrying two mutations in *tat* (pCMV $\Delta$ R 8.93) was then constructed. The first mutation is a deletion of the T in the ATG initiation codon of the *tat* gene; the second is an insertion of a *Mlu*I linker producing a translation stop codon after residue 46 of the Tat protein. These changes confer a *tat*-defective phenotype to HIV-1 (12). After transfection of the control or *tat*-defective packaging constructs into 293T cells, comparable yields of vector particles were recovered in the culture medium, as assayed by using the Gag p24 antigen (see Table 3). Such Tat independence was expected from the replacement of the HIV LTR by the constitutive CMV promoter in the packaging construct. However, the particles produced in the absence of Tat had a dramatically reduced transducing activity (Table 1): 5 to 15% of that of particles produced by the control Tat-positive packaging construct.

We also tested whether the Tat-defective phenotype could be rescued by complementation in target cells (Table 1). HeLa-Tat cells, a cell line expressing Tat from the HIV-1 LTR (13), were transduced by vectors produced with or without Tat. The expression of Tat in target cells did not compensate for the loss in transduction efficiency of vector produced without Tat.

As expected from the Northern analysis, functional inactivation of the *tat* gene resulted in a lower abundance of vector RNA in producer cells. This was indicated by the decrease in luciferase activity in cells producing a luciferase vector without an internal promoter. In this case, transgene expression di-

TABLE 1. Transducing activities of lentivirus vectors made with and without a functional *tat* gene in the packaging construct<sup>a</sup>

Transfer vector	Target cells	Mean transducing activity (TU/ng of p24) $\pm$ SE <sup>b</sup>	
		With <i>tat</i> in packaging construct	Without <i>tat</i> in packaging construct
pHR'CMV-LacZ	293T	1,056 $\pm$ 54	152 $\pm$ 26
pHR2PGK-eGFP	HeLa	5,666	384
pHR'CMV-Luciferase	HeLa	3,000 $\pm$ 152	152 $\pm$ 26
	HeLa-tat	3,777 $\pm$ 348	486 $\pm$ 59
pHR'Luciferase <sup>c</sup>	HeLa	46 $\pm$ 1	0.3 $\pm$ 0.003
	HeLa-tat	3,296 $\pm$ 276	174 $\pm$ 75

<sup>a</sup> Vectors were produced by transfection of the indicated transfer vector, a packaging construct either with (pCMV $\Delta$ R 8.91) or without (pCMV $\Delta$ R 8.93) a functional *tat* gene, and plasmid pMD.G into 293T cells. Serial dilutions of transfected conditioned medium were incubated with the indicated cells, and the cultures were scored after 3 days. For calculating transduction activity, samples were selected from the linear portion of the vector dose-response curve.

<sup>b</sup> LacZ transduction was measured by 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal) staining and by expression of the number of blue cell colonies as a function of the amount of p24 antigen in the inoculum. eGFP transduction was measured by FACS analysis, multiplying the fraction of fluorescent cells by the number of infected cells, and expressing the result as a function of the amount of p24 antigen in the inoculum. Luciferase transduction was measured by luminescence in RLU above background of 50  $\mu$ l of culture extract and dividing the number of RLU  $\times 10^{-2}$  by the number of nanograms of p24 antigen in the inoculum. Means of duplicate (pHR2 PGK-eGFP) or triplicate (all other constructs) determinations are shown.

<sup>c</sup> Without internal promoter.

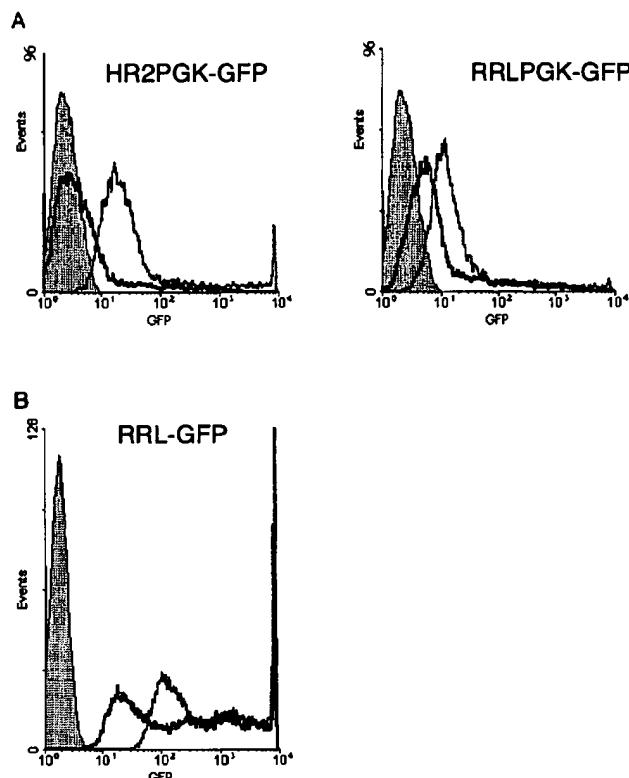


FIG. 2. Transcriptional activities of wild-type and 5' chimeric vector constructs in the absence and presence of Tat. (A) Control pH2 and the 5' chimeric pRRL transfer construct carrying a PGK-eGFP expression cassette were transfected into 293T cells with a packaging construct having a functional (pCMVΔR8.91; grey line) or inactive (pCMVΔR8.93; black line) *tat* gene. GFP expression was analyzed by FACS. The filled area represents nontransfected cells. In the absence of Tat, the chimeric construct yielded a level of GFP expression higher than that achieved by the pH2 construct. Both constructs were further upregulated by Tat, as shown by the increased eGFP expression of cells cotransfected with the control packaging construct. Tat upregulation was proven to be a direct effect by transfecting a pRRL-eGFP vector lacking an internal promoter with control or *tat*-defective packaging constructs and analyzing GFP expression by FACS (Fig. 2B). Comparable results were obtained with the other chimeric LTR vectors (not illustrated). Vector particles were then collected from the transfected producer cells and assayed for transduction of eGFP into HeLa cells and human primary lymphocytes (peripheral blood lymphocytes [PBL]). As shown in Table 2, all vectors had efficient transducing activity, as assessed by endpoint titration on HeLa cells or maximal transduction frequency of PBL. The vector produced by the pRRL chimera was as efficient as that produced by the pH2 construct and was selected to test transduction independent of Tat. As shown in Table 3, the pRRL construct yielded a vector of only slightly reduced transducing activity (60%) when the packaging construct was *tat* defective. The residual effect of

rectly reflects the abundance of transcripts originating from the LTR. 293T cells producing luciferase vectors without Tat had only 5% of the luciferase content of cells producing the same vector with Tat ( $[1.0 \pm 0.2] \times 10^9$  relative light units [RLU]/dish without Tat;  $[20.2 \pm 0.7] \times 10^9$  RLU/dish with Tat). This ratio corresponded very closely to that observed in cells transduced by either type of vector in the course of the same experiment (Table 1), suggesting that the abundance of vector RNA in producer cells is a rate-limiting factor in the transduction by lentivirus vectors.

One could thus conclude that Tat is required in producer cells to activate transcription from the HIV LTR and to generate vector particles with a high transducing activity.

The *tat* requirement is offset by placing a constitutive promoter upstream of the transfer vector. If the only function of Tat is *trans* activation of vector transcription from the LTR, the *tat*-defective phenotype should be rescued by placing a strong constitutive promoter upstream of the vector transcript. Three transcriptional domains have been identified in the HIV promoter in the U3 region of the LTR: the core or basal domain, the enhancer, and the modulatory domain (27). Transcription starts at the U3/R boundary, the first nucleotide of R being numbered 1. The core promoter contains binding sites for the

TATA-binding protein (−28 to −24) and SP-1 (three binding sites between −78 to −45). The enhancer contains two binding sites for NF-κB which overlap with a binding site for NFATc (−104 to −81). The modulatory domain contains binding sites for several cellular factors, including AP-1 (−350 to −293), NFAT-1 (−256 to −218), USF-1 (−166 to −161), Ets-1 (−149 to −141), and LEF (−136 to −125). A panel of 5' chimeric transfer constructs carrying substitutions of either all or part of the U3 region of the 5' LTR was generated. All substitutions were made to preserve the transcription initiation site of HIV. Partial substitutions joined new enhancer sequences to the core promoter of the HIV LTR (−78 to 1), while full substitutions replaced also the promoter. pRLL and pRRL vectors carried the enhancer and the enhancer/promoter, respectively, of RSV; pCCL and pCCL vectors carried the enhancer and the enhancer/promoter of human CMV.

Control pH2 and 5' chimeric transfer constructs carrying a PGK-eGFP expression cassette were tested by transfection of 293T cells with control or *tat*-defective packaging constructs, and the expression of the eGFP transgene was analyzed by fluorescence-activated cell sorting (FACS). The RRL chimeric construct yielded a higher level of eGFP expression than the pH2 vector, reflecting the constitutive transcriptional activity of the new sequence (Fig. 2A). Interestingly, the chimeric vector also displayed upregulation by Tat, as shown by the increased eGFP expression of cells cotransfected with the control packaging construct. Tat upregulation was proven to be a direct effect by transfecting a pRRL-eGFP vector lacking an internal promoter with control or *tat*-defective packaging constructs and analyzing GFP expression by FACS (Fig. 2B). Comparable results were obtained with the other chimeric LTR vectors (not illustrated). Vector particles were then collected from the transfected producer cells and assayed for transduction of eGFP into HeLa cells and human primary lymphocytes (peripheral blood lymphocytes [PBL]). As shown in Table 2, all vectors had efficient transducing activity, as assessed by endpoint titration on HeLa cells or maximal transduction frequency of PBL. The vector produced by the pRRL chimera was as efficient as that produced by the pH2 construct and was selected to test transduction independent of Tat. As shown in Table 3, the pRRL construct yielded a vector of only slightly reduced transducing activity (60%) when the packaging construct was *tat* defective. The residual effect of

TABLE 2. GFP transduction by lentivirus vectors made by transfer constructs with a wild-type or 5' chimeric LTR

Transfer construct	Endpoint titer on HeLa cells (TU/ml) <sup>a</sup>	Transduction efficiency on human lymphocytes (% positive cells) <sup>b</sup>
pH2	$2.3 \times 10^7$	30
pCCL	$4.6 \times 10^6$	14
pCCL	$7.9 \times 10^6$	18
pRRL	$1.8 \times 10^7$	29
pRLL	$8.9 \times 10^6$	18

<sup>a</sup> Determined by multiplying the percentage of fluorescent cells for the vector dilution and the number of infected cells. Samples were selected from the linear portion of the vector dose-response curve.

<sup>b</sup> Percentage of fluorescent human PBL after infection of  $10^6$  cells with 1 ml of vector containing medium. Primary human T lymphocytes were isolated and transduced as previously described (14). Vectors carrying a PGK-eGFP expression cassette were produced by transfection of the indicated transfer construct, the packaging plasmid pCMVΔR8.91, and the envelope plasmid pMD.G into 293T cells. Fluorescent cells were scored by FACS analysis 6 days after transduction. Data are averages of duplicate determinations for a representative experiment of three performed.

TABLE 3. GFP transduction into HeLa cells by lentivirus vectors made by transfer constructs with a wild-type or 5' chimeric LTR and packaging constructs with or without a functional *tat* gene<sup>a</sup>

Transfer construct	<i>tat</i> gene in packaging construct	Endpoint titer (TU/ml)	p24 antigen (ng/ml)	Transduction efficiency (TU/ng of p24)
pHR2	+	4.1 × 10 <sup>6</sup>	297	13,805
pHR2	-	2.4 × 10 <sup>5</sup>	545	440
pRRL	+	1.3 × 10 <sup>7</sup>	546	23,810
pRRL	-	4.9 × 10 <sup>6</sup>	344	14,244

<sup>a</sup> Vectors carrying a PGK-eGFP expression cassette were produced by transfection of the indicated transfer and packaging plasmid plus plasmid pMD.G into 293T cells. Serial dilutions of transfectant conditioned medium were incubated with HeLa cells, and the cultures were scored after 6 days. For calculating endpoint titers, samples were selected from the linear portion of the vector dose-response curve. Data are averages of duplicate determinations for a representative experiment of five performed.

Tat on transduction was in agreement with the ability of Tat to upregulate transcription from the chimeric LTR.

The use of the chimeric LTR construct allowed removal of Tat from the packaging system with a minimal loss in the

transduction efficiency of the vector in vitro. To test vector performance in the more challenging setting of in vivo delivery into brain neurons, high-titer vector stocks were generated from the pHR2 and pRRL constructs with and without Tat. The four stocks of eGFP vector were matched for particle content by p24 antigen and injected bilaterally in the neostriata of groups of three adult rats. The animals were sacrificed after 1 month, and serial sections of the brain were analyzed for eGFP fluorescence (not shown) and immunostained by antibodies against eGFP (Fig. 3). The results obtained in vivo matched the in vitro data. Vector produced by the pHR2 construct only achieved significant transduction of the neurons when packaged in the presence of Tat. Vector produced by the pRRL chimera was as efficient when made with or without Tat. The transduction extended throughout most of the striatum and reached a very high density of positive cells in the sections closest to the injection site. No signs of pathology were detectable in the injected tissue, except for a small linear scar marking the needle track, by hematoxylin and eosin staining of the sections (data not shown).

These results provide evidence that Tat is dispensable for efficient transduction by a lentivirus vector.

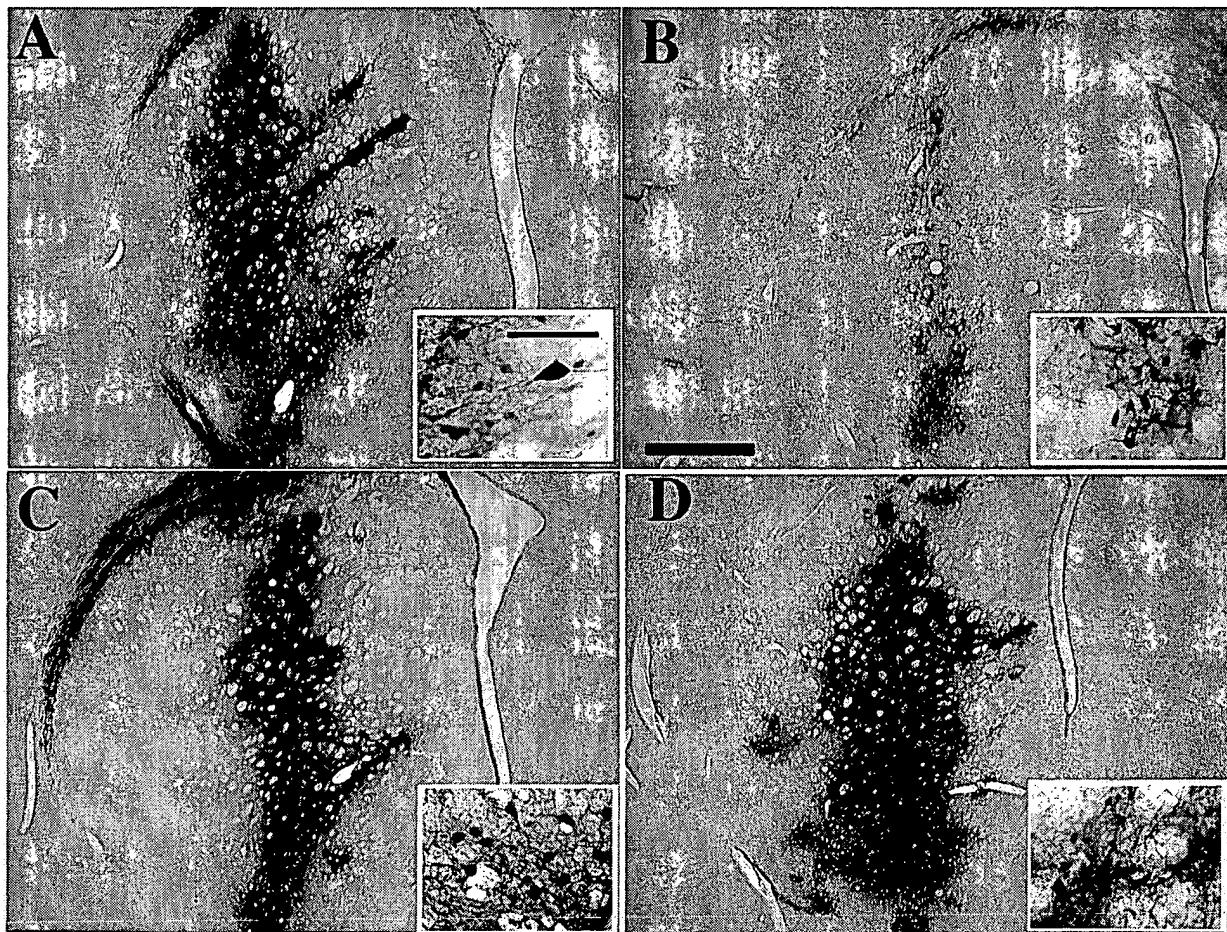


FIG. 3. In vivo transduction of eGFP into brain cells by lentivirus vectors produced with and without Tat. Vectors carrying a PGK-eGFP expression cassette were produced by the pHR2 (A and B) or the 5' chimeric pRRL (C and D) transfer construct and a packaging construct with (pCMVAR8.91; A and C) or without (pCMVΔR8.93; B and D) a functional *tat* gene, concentrated by ultracentrifugation, and normalized for particle content prior to injection into the corpora striata of adult rats. One month after injection, brain sections were stained for immunoreactivity to the GFP protein. While both types of vectors transduced neurons very efficiently when made with Tat, only the vector made by the chimeric transfer construct worked as well when produced without Tat. Representative sections close to the injection site are shown for one of six striata injected per each type of vector. The bar in panel B represents 1 mm; that in the inset in panel A represents 100 μm.

TABLE 4. GFP transduction into HeLa cells by lentivirus vectors made by linked or split packaging constructs and a pRRL transfer construct<sup>a</sup>

Packaging construct	Separate <i>rev</i> plasmid <sup>b</sup>	p24 antigen (ng/ml)	Endpoint titer (TU/ml)	Transduction efficiency (TU/ng of p24)
pCMVΔR8.74		364	1.07 × 10 <sup>7</sup>	29,436
pMDLg/pRRE		<0.1	ND	NA
pMDLg/pRRE	TK-Rev, 5 µg	29	6.9 × 10 <sup>5</sup>	23,793
pMDLg/pRRE	TK-Rev, 12 µg	94	2.02 × 10 <sup>6</sup>	21,489
pMDLg/pRRE	RSV-Rev, 2.5 µg	774	1.0 × 10 <sup>7</sup>	13,495
pMDLg/pRRE	RSV-Rev, 5 µg	776	7.6 × 10 <sup>6</sup>	9,761
pMDLg/pRRE	RSV-Rev, 12 µg	565	4.8 × 10 <sup>6</sup>	8,495

<sup>a</sup> Vectors carrying a PGK-eGFP expression cassette were produced by the transfection of a self-inactivating pRRL transfer construct (with a deletion in the 3' LTR [53]), the indicated packaging and *rev* plasmids, and plasmid pMD.G into 293T cells. Serial dilutions of transfected conditioned medium were incubated with HeLa cells, and the cultures were scored after 6 days. For calculating endpoint titers, samples were selected from the linear portion of the vector dose-response curve. Data are averages of duplicate determination for a representative experiment of three performed. ND, none detected (the detection limit of the assay was 10<sup>2</sup> TU/ml); NA, not applicable.

<sup>b</sup> The promoter driving the expression of a synthetic *rev* cDNA and the amount of plasmid transfected are indicated.

**A new split-genome conditional packaging system.** The possibility of deleting the *tat* gene prompted us to explore a new design of the packaging component of the HIV vector system, in which two separate nonoverlapping expression plasmids, one for the *gag* and *pol* genes and the other for the *rev* gene, were used. The *gag* and *pol* reading frames were expressed within the context of the MD cassette, which employs the CMV promoter and intervening sequence and the human β-globin poly(A) site (34). All HIV sequences upstream of the *gag* initiation codon were removed, and the leader was modified for optimal fit to the Kozak consensus for translation. This construct, however, expressed almost no p24 antigen when transfected alone in 293T cells. This observation is in agreement with the previously reported presence of *cis*-repressive or inhibitory sequences in the *gag* and *pol* genes (40, 41). The HIV RRE was then inserted downstream of the *pol* gene, and the resulting plasmid was cotransfected with a *rev* expression vector (Table 4). High levels of p24 antigen production were observed in this case, the highest yields being obtained when *rev* was driven by an RSV promoter. When the *gag-pol* and the *rev* constructs were cotransfected with the pRRL chimeric transfer vector and the VSV G-expressing plasmid, high-titer vector was obtained in the culture medium. Both the yield of particles and their transducing efficiency were similar to those obtained with previous versions of the system. Northern analysis of producer cells confirmed that unspliced vector genomic RNA accumulated only in the presence of Rev (data not shown). Thus, both the expression of the *gag* and *pol* genes and the accumulation of packageable vector transcripts are dependent on *trans* complementation by a separate Rev expression construct. Such a conditional packaging system provides an important safety feature unavailable to oncoretrovirus vectors.

## DISCUSSION

The predicted biosafety of a viral vector depends in part on how much segregation of the *cis*- and *trans*-acting functions of the viral genome is achieved by the vector design and is maintained during vector production. A vector particle is assembled by viral proteins expressed in the producer cell from a construct(s) stripped of the *cis*-acting sequences required for the transfer of the viral genome to target cells (packaging con-

struct). These *cis*-acting sequences are instead linked to the transgene in the transfer vector. As the vector particle packages only the genetic information contained in this latter construct, the infection process is limited to a single round without spreading. Through recombination, it is possible that sequences encoding viral proteins rejoin the *cis*-acting elements of the transfer vector. If the resulting recombinant expresses all required functions, it is able to replicate (i.e., it is a replication-competent retrovirus [RCR]) and presents a risk to the recipient. The formation of heterozygous vector particles containing RNAs from both the packaging and transfer vectors, followed by homologous recombination during reverse transcription, is the mechanism most often incriminated in the emergence of RCR during the production of retroviral vectors. The likelihood of this type of recombination is dependent on residual *cis*-acting sequences in the packaging plasmid, allowing some level of encapsidation, and on the extent of homology between packaging and vector constructs (10).

A first strategy to improve the biosafety of a vector is to use nonoverlapping split-genome packaging constructs that require multiple recombination events with the transfer vector for RCR generation. Earlier studies described several approaches to generate replication-defective HIV vectors (7, 35, 38, 42). However, these vectors could be produced only to low infectious titers, were restricted to CD4-positive cellular targets, and carried the risk of generating wild-type HIV by recombination of the components. A major advance was achieved when an improved vector design was combined with the use of the envelope of another virus (32, 33, 39). The lentivirus vector that we describe here is packaged by three nonoverlapping expression constructs, two expressing HIV proteins and the other expressing the envelope of a different virus. Moreover, all HIV sequences known to be required for encapsidation and reverse transcription (2, 22, 24, 27, 29, 30, 35) are absent from these constructs, with the exception of the portion of the *gag* gene that contributes to the stem-loop structure of the HIV-1 packaging motif (29).

A second strategy to improve vector biosafety took advantage of the complexity of the lentivirus genome. The minimal set of HIV-1 genes required to generate an efficient vector was identified, and all other HIV reading frames were eliminated from the system. As the products of the removed genes are important for the completion of the virus life cycle and for pathogenesis, no recombinant can acquire the pathogenetic features of the parental virus. We previously demonstrated that all four accessory genes of HIV could be deleted from the packaging construct without compromising gene transduction (51). In this work, we went further by deleting another factor crucial for HIV replication, the *tat* gene. Its product is one of the most powerful transcriptional activators known and plays a pivotal role in the exceedingly high replication rates that characterize HIV-induced disease (18, 19, 47).

It was found that Tat was required in producer cells to generate vector of efficient transducing activity but that this requirement was offset by inducing constitutive high-level expression of vector RNA. Due to the low basal transcription from the HIV LTR, Tat was necessary to increase the abundance of vector transcripts and allow their efficient encapsidation by the vector particles. When made in the absence of Tat, vector particles had 10- to 20-fold-reduced transducing activity. However, when strong constitutive promoters replaced the HIV sequence in the 5' LTR of the transfer construct, vectors made without Tat exhibited a less than twofold reduction in transducing activity. As Tat strongly upregulated transcription from the chimeric LTR, the transducing activity of the output particles must reach saturation. The abundance of vector RNA

in producer cells thus appears to be a rate-limiting factor for transduction until it reaches a threshold. Conceivably, an upper limit is set by the total output of particles available to encapsidate vector RNA. As the total particle output varied with the types of vector and internal promoter used, this may explain the quantitative differences obtained in response to *tat* deletion.

Successful deletion of the *tat* gene was unexpected in view of a reported additional role for Tat in reverse transcription (17, 20). While the reasons for this discrepancy are not obvious, it should be noted that the transduction pathway of the lentivirus vector mimics only in part the infection pathway of HIV. The vector is pseudotyped by the envelope of an unrelated virus and contains only the core proteins of HIV, without any accessory gene product. The VSV envelope targets the vector to the endocytic pathway, and it has been shown that redirection of HIV-1 from its normal route of entry by fusion at the plasma membrane significantly changes the biology of the infection. For example, Nef and cyclophilin A are required for the optimal infectivity of wild-type HIV-1 but not of a (VSV G) HIV pseudotype (1). It is also possible that the kinetics of reverse transcription are more critical for the establishment of viral infection than for gene transduction, given the differences in size and sequence between the virus and vector genome.

Tat-independent transduction by an HIV-based vector was recently reported by Kim et al. for *in vitro* cellular targets (23). In the vector designed by these authors, however, Tat and Rev were expressed from the transfer vector and thus were also present in target cells. A CMV-HIV hybrid LTR was used; this construct yielded vector titers approximately 30% of that obtained with an intact LTR. When the *tat* gene was inactivated, the titer did not change. Srinivasakumar et al. (43) previously reported a rather low (5- to 10-fold) dependence on Tat of an HIV-based vector produced by cells stably expressing the HIV structural proteins. In this case, titers of  $5 \times 10^3$  TU/ml with Tat and  $7 \times 10^2$  TU/ml without Tat were obtained on HeLa-CD4 cells. Although these titers are much lower than those reported here, the vector particles carried the HIV envelope, an indication that Tat is not absolutely required for transduction by vector particles which in that case mirror more closely the wild-type virus. It remained possible, however, that a dependence on Tat may be revealed in more challenging gene deliveries into the body tissues that are the actual targets of gene therapy. This could have been due to a stricter Tat requirement for optimal transduction efficiency or for the production of high-titer vector stocks or to differences in cell-type-specific factors. Our results now establish that Tat is fully dispensable for lentivirus vector transduction even when high titers are achieved and, most importantly, for gene delivery *in vivo* into terminally differentiated neurons of an adult rat brain.

The Northern analysis of producer and target cells shows that the Tat dependence of LTR-driven expression restricts the production of vector genomic RNA to producer cells. This applies as well to vectors made by the 5' chimeric constructs, as the U3 sequences of both LTRs of the resulting provirus are derived from the vector 3' LTR. However, the functional replacement of the *tat* gene in the packaging construct by promoter sequences upstream of the transfer construct makes the generation of a transcriptionally active recombinant much more unlikely. This will be even more significant in stable producer cell lines that avoid the risk of plasmid recombination during cotransfection.

We also exploited the Rev dependence of *gag-pol* expression and of the accumulation of unspliced, packageable transcripts. Yu et al. (50) previously showed that the dependence on Rev can be used to make expression of HIV genes inducible. We

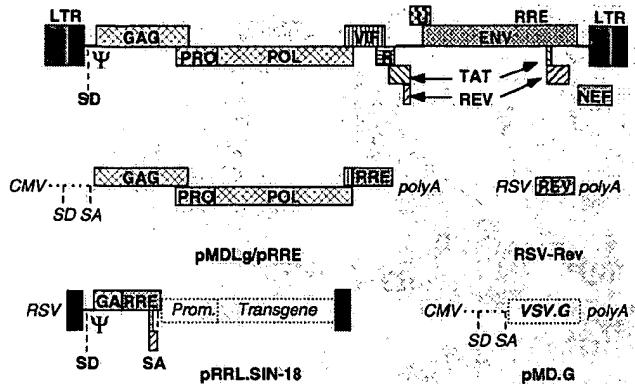


FIG. 4. Schematic drawing of the HIV provirus and the four constructs used to make a lentivirus vector of the third generation. The viral LTRs, the reading frames of the viral genes, the major 5' splice donor site (SD), the packaging sequence ( $\Psi$ ), and the RRE are boxed and indicated in bold type. The conditional packaging construct, pMDLg/pRRE, expresses the *gag* and *pol* genes from the CMV promoter and intervening sequences and polyadenylation site of the human  $\beta$ -globin gene. As the transcripts of the *gag* and *pol* genes contain *cis*-repressive sequences, they are expressed only if Rev promotes their nuclear export by binding to the RRE. All *tat* and *rev* exons have been deleted, and the viral sequences upstream of the *gag* gene have been replaced. A nonoverlapping construct, RSV-Rev, expresses the *rev* cDNA. The transfer construct, pRRL.SIN-18, contains HIV-1 *cis*-acting sequences and an expression cassette for the transgene. It is the only portion transferred to the target cells and does not contain wild-type copies of the HIV LTR. The 5' LTR is chimeric, with the enhancer/promoter of RSV replacing the U3 region (RRL) to rescue the transcriptional dependence on Tat. The 3' LTR has an almost complete deletion of the U3 region, which includes the TATA box (from nucleotides -418 to -18 relative to the U3/R border). As the latter is the template used to generate both copies of the LTR in the integrated provirus, transduction of this vector results in transcriptional inactivation of both LTRs; thus, it is a self-inactivating vector (SIN-18). The fourth construct, pMD.G, encodes a heterologous envelope to pseudotype the vector, here shown coding for VSV G. Only the relevant parts of the constructs are shown.

describe a core packaging system split in two separate nonoverlapping expression constructs, one for the *gag* and *pol* reading frames optimized for Rev-dependent expression and the other for the *rev* cDNA. This third-generation packaging system matches the performance of its predecessors in terms of both yield and transducing efficiency. However, it increases significantly the predicted biosafety of the vector. It has been suggested that the Rev-RRE axis could be replaced by the use of constitutive RNA transport elements of other viruses, although at the price of decreased efficiency (11, 23, 43). We would suggest that maintaining the Rev dependence of the system allows for an additional level of biosafety through the splitting of the HIV-derived components of the packaging system.

The conditional packaging system described here can be combined with a self-inactivating vector construct carrying a major deletion in the 3' LTR (52). This vector design (Fig. 4) offers significant biosafety features. The contribution of HIV is reduced to a fraction of *cis*-acting sequences in the vector, leaving out in particular most of the LTR, and to only three genes, *gag*, *pol*, and *rev*, in the packaging constructs, compared with the nine genes necessary for the *in vivo* replication and pathogenesis of wild-type HIV-1 (3, 18, 27, 49). The actual biosafety of a vector must be proven *in vivo*. However, given the serious limitations of the available animal models of HIV-induced disease, the biosafety of HIV-derived vectors will ultimately be proven only in human hosts. Therefore, the vector design must ensure the highest predictable biosafety for clinical testing to be acceptable.

It is noteworthy that the fraction of the HIV-1 genome that is left in the vector is probably smaller than could be achieved

with any of the nonprimate lentiviruses, the genomic complexity of which is lower than that of HIV-1 (37). Also, the risks associated with the introduction in humans of a recombinant arising from a nonprimate lentivirus, even in a form that in its cognate animal species appears to be attenuated, are very difficult to assess, as illustrated by the ongoing debate on xenotransplantation (48). In contrast, the almost two decades spent studying a virus that has now spread in tens of millions of people worldwide have revealed a considerable amount of information on the pathogenic features of HIV-1, in particular on the dependence of virulence on a crucial set of viral genes. Based on these data, we would like to suggest that the HIV-based vectors described here are good candidates for the clinical trial of lentivirus vectors in human gene therapy.

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VIRAL TRANSFER TECHNOLOGY

HOT TECHNIQUE

# Improved titers of HIV-based lentiviral vectors using the SRV-1 constitutive transport element

MR Mautino, N Keiser and RA Morgan

Clinical Gene Therapy Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, USA

*The development of lentiviral vectors that use Rev-independent mechanisms of nuclear export for their genomic RNA could facilitate the construction of novel anti-HIV vectors. We have improved the titers of Rev-independent lentiviral vectors having the SRV-1 CTE by mutating the major splice donor and acceptor sites present in the vector and by relocalization of the CTE sequences adjacent to the HIV-1*

*3'LTR. These two modifications have additive beneficial effects on vector titers and packaging efficiency. Packaging these CTE<sup>+</sup> vectors expressing marker genes with a Rev-dependent HIV-1 helper vector yields higher titers than are obtained using a Rev-dependent lentiviral vector. Gene Therapy (2000) 7, 1421-1424.*

**Keywords:** Rev-independent; lentiviral vector; CTE; gene therapy; HIV/AIDS

The use of HIV-based conditionally replicating vectors expressing anti-HIV genes for the treatment of AIDS has several theoretical advantages over the current vector systems being employed.<sup>1</sup> Even in the absence of specific inhibitory genes, these vectors would be able to inhibit HIV replication by acting as decoys for the HIV regulatory proteins Tat and Rev, and by competing for packaging into HIV-1-encoded virions, facilitating the spread of the vector to unprotected cells *in vivo*.<sup>2-5</sup> In order to provide the most effective inhibition of HIV replication, the vector must have a competitive advantage for packaging over the wild-type virus mRNA. To achieve this when an anti-HIV gene is inserted into the vector, the vector must be less sensitive to the anti-HIV gene than wild-type virus.

Some of the most potent anti-HIV genes characterized so far are transdominant negative mutants of Rev.<sup>6,7</sup> Expressing such anti-HIV genes in HIV-based vectors would require the use of a Rev-independent system to produce significant quantities of vector. Several attempts have been made to develop Rev-independent lentiviral vectors based on HIV-1 or SIV-1.<sup>8-13</sup> These systems make use of the MPMV or SRV-1 constitutive transport element (CTE) to complement for the absence of the RRE/Rev mRNA transport mechanism. Most studies have focused on the use of the CTE to increase the expression of Gag/Pol from helper plasmids lacking RRE sequences, while using transfer vectors that are dependent on the RRE/Rev mRNA transport mechanism. In these cases the synthesis of Gag/Pol was reduced seven-fold,<sup>8</sup> to 50-fold<sup>9</sup> relative to those using the RRE/Rev regulatory mechanism, resulting in vector titers that were 10<sup>4</sup> and 10<sup>3</sup>

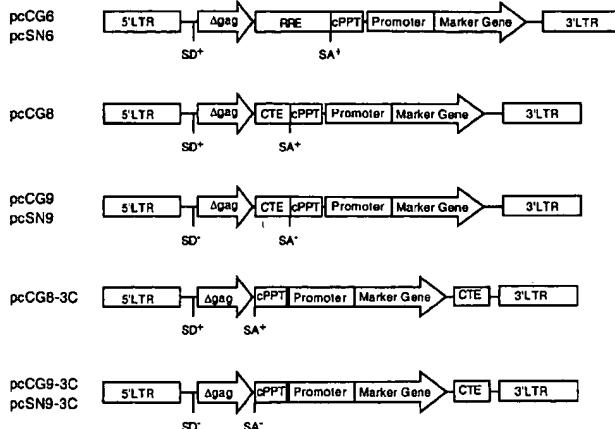
cfu/ml, respectively. In the best reported case, titers of 10<sup>5</sup> cfu/ml have been obtained using an analogous combination vector system.<sup>10</sup>

In a previous report, we explored the reciprocal combination system, in which the RRE sequences in the transfer vector were replaced by the SRV-1 CTE while using a fully Rev-dependent helper vector.<sup>14</sup> It has been argued that the presence of the RRE in the transfer vector rather than in the helper vector would increase the safety of the vector as recombination events occurring during packaging would produce transfer vectors retaining the RRE that would be incompetent for replication in the absence of Rev. However, in conditionally replicating lentiviral vectors designed to express TdRev, the Rev-independent character of the vector has to reside in the transfer vector itself rather than in the helper vector. This report's goal was to design the most optimal CTE<sup>+</sup> Rev-independent lentiviral vector.

An example of a fully Rev-dependent vector is pcCG6 (Figure 1). This vector contains an RRE element cloned within an intron, expresses EGFP under the control of the CMV promoter and can produce biologically active titers in excess of 10<sup>6</sup> cfu/ml.<sup>14</sup> Cotransfection of this plasmid with the Rev-dependent helper pCMVΔR8.2<sup>15</sup> produces vector preparations capable of transducing 27% of the target cells (Figure 2a). As we previously reported, replacing the RRE sequences by a 280-bp fragment containing the SRV-1 CTE (vector pcCG8), results in a 15-fold reduction in vector titers (Figure 2a,  $P < 0.001$ ). This was consistent with the reduction in vector titers observed by others when introducing the CTE in one of the components of the packaging system. As the function of the CTE is to increase the transport of unspliced cytoplasmic mRNAs by facilitating their nuclear export rather than by preventing their splicing,<sup>16</sup> we reasoned that we might be able to increase vector titers by augmenting the fraction of unspliced cytoplasmic mRNA available for packaging. This was accomplished by mutating the major

Correspondence: R Morgan, Clinical Gene Therapy Branch, NHGRI, 10 Center Drive, Building 10, Room 10C103, Bethesda, MD 20892-1851, USA

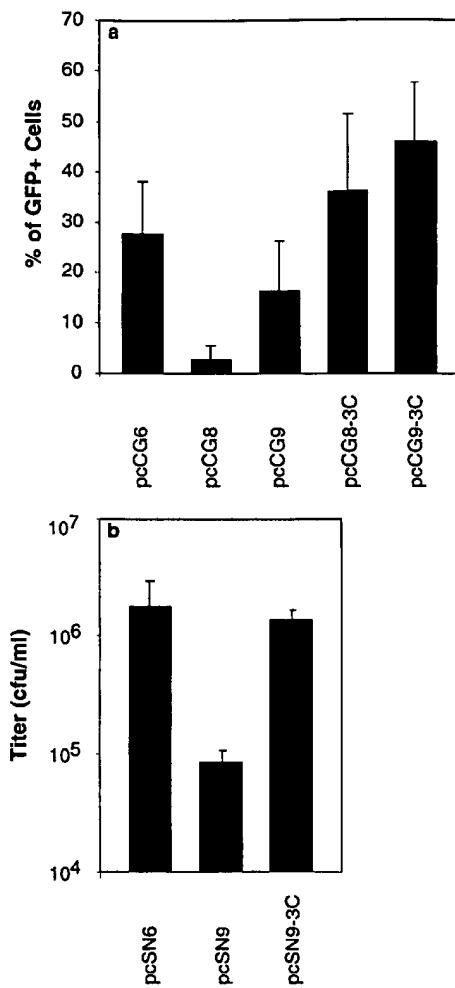
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**Figure 1** Schematic structures of the vectors used in this study. Construction of vector pcCG6, pcSN6, pcCG8, pcCG9, pcSN9 has been described in Mautino et al.<sup>14</sup> Vectors pcCG8-3C and pcCG9-3C were constructed by deleting a 280 bp *Xba*I fragment containing the SRV-1 CTE sequences from pcCG8 and pcCG9, and cloning it back into an *Avr*II site downstream of the EGFP gene. To create plasmid pcSN9-3C, a *Spe*I fragment encompassing the CMV-EGFP expression cassette from pcCG9-3C was deleted and replaced by an *Spe*I-*Xba*I fragment containing the SV40-Neo expression cassette obtained by PCR from plasmid pcSN7.<sup>14</sup> The following features are depicted in the vector structures: LTR, HIV-1 long terminal repeat; Δagag, HIV-1 gag gene truncated at the *Nsi*I site of HIV-1NL4-3; RRE, Rev responsive element; cPPT, central polypurine tract; CTE, SRV-1 constitutive transport element; SD<sup>+</sup>/SA<sup>+</sup>, wild-type splicing donor and acceptor sites; SD<sup>-</sup>/SA<sup>-</sup>, mutant splicing donor and acceptor sites. In the vector names, CG indicates a CMV-EGFP expression cassette, while SN indicates an SV40-Neo expression cassette.

splice donor and acceptor sites that were flanking the CTE, creating vector pcCG9<sup>14</sup> which showed a six-fold increase in the number of transduced cells with respect to pcCG8 (Figure 2a,  $P < 0.01$ ). In spite of this improvement, vector titers were approximately  $7 \times 10^5$  cfu/ml, which was still five-fold lower than a fully Rev-dependent vector system.

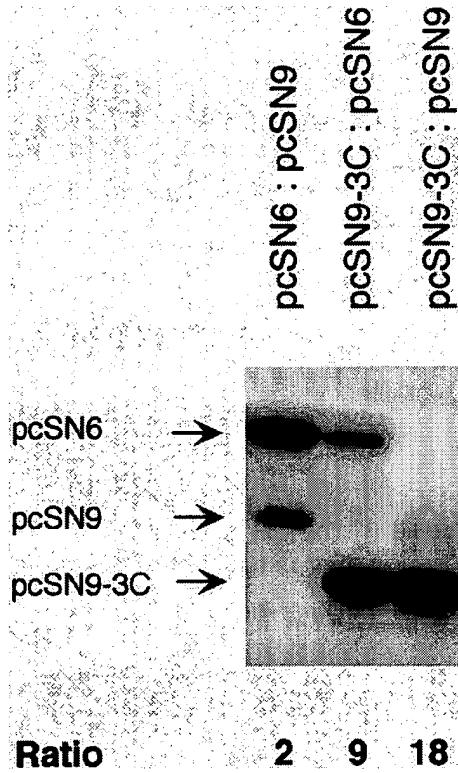
It has been reported that the MPMV CTE functions in a position-dependent manner in the context of replication-incompetent SIV-1 vectors.<sup>17</sup> These experiments indicated that the function of CTE decreases as the distance between the CTE and the poly(A) sequences increases beyond 200 bp. As the distance between the CTE and the poly(A) site in the 3'HIV LTR in our vector pcCG8 and pcCG9 is 2390 bp, we tested whether moving the CTE closer to the poly(A) site would improve vector titers. To do this we removed the fragment containing the CTE sequences from pcCG8 and pcCG9 and cloned it downstream of the EGFP gene to create vectors pcCG8-3C and pcCG9-3C (Figure 1). This reduced the distance between the CTE and the poly(A) site to 730 nt. Figure 2a shows that the percentage of GFP<sup>+</sup> cells that can be obtained with these vectors in parallel transfection and transduction assays is 12-fold ( $P < 0.01$ ) and three-fold ( $P < 0.01$ ) higher for pcCG8-3C and pcCG9-3C than for pcCG8 and pcCG9, respectively. This suggests that moving the CTE to a location closer to the poly(A) site was more beneficial to improve vector function than mutating the splicing signals. Moreover, the combination of these two modifications has an additive effect, allowing the increase of titers to levels greater than observed with a fully Rev-dependent vector system such as pcCG6 (1.6-fold;  $P < 0.02$ ).



**Figure 2** Influence of structural modifications on vector titers. 10  $\mu$ g of each vector plasmid DNA were cotransfected with 7  $\mu$ g of helper plasmid pCMVAR8.2,<sup>15</sup> 3  $\mu$ g of plasmid LTR-G encoding the VSV-G envelope protein, and 1  $\mu$ g of plasmid pGL3 encoding luciferase to control for transfection efficiency, into  $4 \times 10^6$  293T cells seeded in 100 mm poly-D-lysine coated dishes. Sixty hours after transfection, supernatants were passed through 0.45  $\mu$ m filters and used to transduce  $3 \times 10^5$  TE671 cells in medium supplemented with 8  $\mu$ g/ml polybrene. For vectors containing the EGFP marker gene (a), 1 ml of undiluted supernatant was used for the transductions, and cells were analyzed by FACS 36 h after transduction. For cells having the Neo marker gene (b), 1:10 serial dilutions of the filtered supernatant were used to transduce TE671 cells, which were selected in DMEM supplemented with G418 1 mg/ml for 12–14 days. Bars represent the averages and standard deviations for five values (a) or seven values (b), respectively.

We next switched the CMV-EGFP expression cassette for an SV40-Neo expression cassette in pcCG9-3C, as this marker permits quantification of the colony-forming units present in the vector supernatant. This created vector pcSN9-3C (Figure 1). We compared the vector titers of pcSN9-3C with the previously described vectors pcSN6 and pcSN9 that have the same SV40-Neo expression cassette.<sup>14</sup> Moving the CTE closer to the 3'HIV LTR produces a 17-fold increase in vector titers (Figure 2b,  $P < 0.01$ ). These titers ( $1.4 \times 10^6$  cfu/ml) are very close to the titers that can be obtained with a fully Rev-dependent vector such as pcSN6 ( $1.8 \times 10^6$  cfu/ml). In order to make a direct comparison of the vector titer

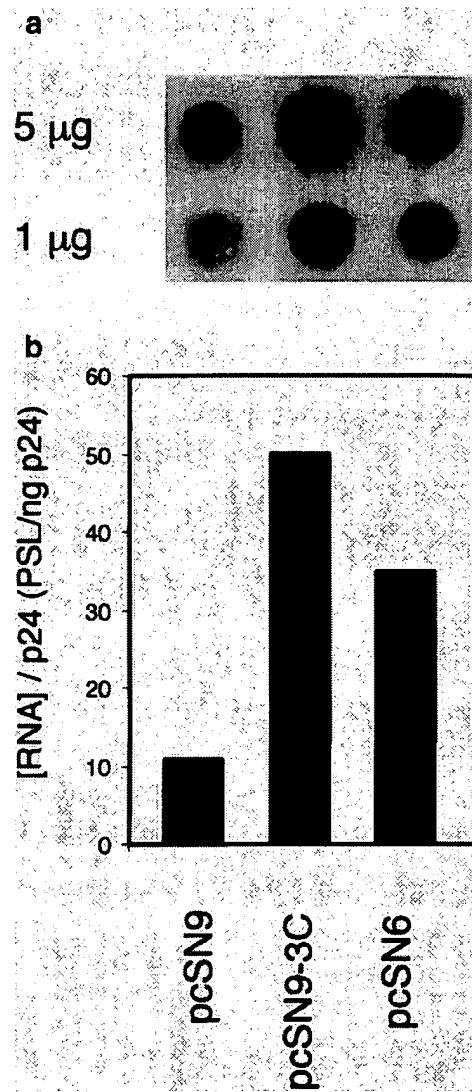
differences between pcSN6, pcSN9 and pcSN9-3C we performed a packaging competition experiment by a two-vector cotransfection (Figure 3). The resultant supernatant was used to transduce target cells from which specific DNA fragments were amplified by PCR from their genomic DNA. The relative intensity of the signals amplified from each vector sequence gives an estimation of the relative input titers that can be obtained with each vector (in the same transfection and transduction events). The data suggest that vector pcSN9-3C is packaged and transduced with an efficiency that has about nine-fold higher than the fully Rev-dependent vector pcSN6, and 18-fold higher than vector pcSN9. An interesting observation is that in these two-vector competition experiments the overall titer on TE671 cells was not affected with respect to what is observed when transfecting one vector at a time (not shown). This suggests that



**Figure 3** Comparison of vector performances by two-vector packaging competition. The two vectors indicated at the top of each lane were cotransfected (5  $\mu$ g of each) along with 8  $\mu$ g of the helper plasmid pCMVΔR8.2 and 3  $\mu$ g of a plasmid encoding the VSV-G envelope into  $4 \times 10^6$  293T cells seeded in a 100 mm dish. After 60 h, the supernatant was filtered and used to transduce TE671 cells. After a 2-week selection in medium supplemented with G418 1 mg/ml, genomic DNA was purified from the transduced TE671 cells and the relative vector copy number was measured by semiquantitative radioactive PCR. The primers used (5' TTAAGGCCAGGGGGAAAGAAACAAT 3', 5' GAGTTAGGG CGGGACTATGGTT 3') recognize the same sequences in vectors pcSN6, pcSN9 or pcSN9-3C, and generate PCR fragments of 1693 bp, 1189 bp and 933 bp, respectively. PCR amplification was performed for 26 cycles (30 s at 94°C, 30 s at 59°C and 3 min at 72°C) in the presence of 10  $\mu$ M  $^{32}$ P- $\alpha$ -dCTP. The radioactive PCR products were electrophoresed through 1% agarose gels and the radioactive signal was quantified using a phosphoimager. The arrows indicate the PCR bands that are amplified from each of the indicated proviruses. The average ratios ( $n = 4$ ) between the intensities of the two bands in each lane, are indicated at the bottom of each lane.

the presence of heterozygous virions does not reduce the overall functionality of the vector preparations.

We then asked whether the efficiency of particle formation was affected by this modification. To assess this, we purified virion mRNA from equal aliquots of vector supernatant and measured the amount of DNase-treated



**Figure 4** Comparison of packaging efficiency between vectors pcSN9, pcSN9-3C and pcSN6. (a) Dot blot hybridization of vector RNA purified from the supernatant. To purify vector RNA, 100  $\mu$ g of carrier yeast tRNA and 1  $\mu$ g of tracer plasmid pEGFP-N1 (Clontech, Palo Alto, CA, USA) were added to 2.5 ml of filtered vector supernatant prepared as described in Figure 2. RNA was extracted with Trizol LS (Gibco BRL, Rockville, MD, USA):chloroform, precipitated from the aqueous phase with isopropanol, resuspended in water, treated with RNase-free DNase, and subjected to another round of extraction with Trizol LS:chloroform. RNA concentration was measured by  $A_{260}$  and 5  $\mu$ g and 1  $\mu$ g of RNA was dot blotted onto nylon membranes and hybridized with a radiolabeled probe corresponding to the LTR-gag region of all vectors. A parallel dot blot was hybridized with an EGFP probe to verify the complete elimination of DNA during the RNA purification process. One representative dot blot of three experiments is shown. (b) The packaging efficiency of each vector was estimated as the ratio between the RNA present in the virions normalized to the total amount of capsids present in the same supernatant. The amount of capsids was estimated by p24 ELISA (Coulter, Fullerton, CA, USA).

vector RNA by dot blot hybridization with a probe complementary to the HIV LTR (Figure 4a). Control for absence of DNA contamination was performed by measuring the elimination of a reporter plasmid that was added to the supernatant before starting the viral RNA purification. Quantification of the hybridization signals indicates that the viral RNA levels of pcSN9-3C are two-fold the levels seen for the Rev-dependent vector pcSN6, and six-fold the levels of RNA observed for pcSN9. When these values were normalized for the total number of capsids measured as p24 present in the supernatant, the results indicate that vector pcSN9-3C has a higher packaging efficiency than vector pcSN6 (1.4-fold) or pcSN9 (five-fold) (Figure 4b). This might reflect higher levels of unspliced genomic RNA available for packaging, either as a result of an enhanced nuclear export efficiency or an enhanced vector genomic RNA stability.

In summary, we have improved the titers of Rev-independent CTE-based lentiviral transfer vectors by mutating the splicing signals and locating the CTE elements closer to the 3' end of the vector transcript, and by packaging them with an HIV-1 helper that uses the RRE/Rev regulatory mechanism to drive the expression of Gag/Pol. The titers obtained with this combination packaging system are of the same level or even higher than the titer that can be obtained with a fully Rev-dependent packaging system. Here, the transfer and helper genomes share homology only in the truncated gag region that forms part of the packaging signal of the transfer vector (468 nt) and thus they should be less prone to generate replication competent vectors than packaging systems in which both the helper and transfer vectors share homology in the RRE region. Moreover, this system gives approximately 15-fold higher titers than the best reciprocal combination packaging system in which the CTE is used to increase expression of Gag/Pol from the helper plasmid and the transfer vector retains the RRE/Rev regulatory elements.

A lentiviral vector whose genomic mRNA does not require Rev for transport to the cytoplasm would possess a competitive advantage for packaging with respect to the genomic RNA of wild-type HIV-1 in cells where TdRev is being expressed by the vector. Such TdRev-expressing lentiviral vectors may be an extremely potent anti-HIV gene therapy system.

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## The Effect of Viral Regulatory Protein Expression on Gene Delivery by Human Immunodeficiency Virus Type 1 Vectors Produced in Stable Packaging Cell Lines

NARASIMHACHAR SRINIVASAKUMAR,<sup>†</sup> NATHALIE CHAZAL, C. HELGA-MARIA,  
SUSAN PRASAD, MARIE-LOUISE HAMMARSKJÖLD, AND DAVID REKOSH\*

*Myles H. Thaler Center for AIDS and Human Retrovirus Research and Department of  
Microbiology, University of Virginia, Charlottesville, Virginia 22908*

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We describe the generation of stable human immunodeficiency virus type 1 (HIV-1)-packaging lines that constitutively express high levels of HIV-1 structural proteins in either a Rev-dependent or a Rev-independent fashion. These cell lines were used to assess gene transfer by using an HIV-1 vector expressing the hygromycin B resistance gene and to study the effects of Rev, Tat, and Nef on the vector titer. The Rev-independent cell lines were created by using *gag-pol* and *env* expression vectors that contain the Mason-Pfizer monkey virus (MPMV) constitutive transport element (CTE). Vector titers approaching  $10^4$  CFU/ml were routinely obtained with these cell lines, as well as with the Rev-dependent cell lines, with HeLa-CD4 cells as targets. The presence of Nef and Tat in the producer cell each increased the vector titer 5- to 10-fold. Rev, on the other hand, was absolutely essential for gene transfer, unless the MPMV CTE was present in the vector. In that case, by using the Rev-independent cell lines for packaging, Rev could be completely eliminated from the system without a reduction in vector titer.

Retroviral vectors based on oncoviruses, such as Moloney murine leukemia virus, have been used extensively for gene transfer with efficient packaging cell lines and vectors developed for this purpose (35). However, a major drawback in the use of these systems is that at least one round of cell division is required for proviral integration into the target cell (36, 47, 53). Thus, these vectors cannot be used for gene transfer to nondividing or growth-arrested cells (36). This has severely restricted their potential usefulness.

In contrast to the simpler oncornaviruses, the more complex human immunodeficiency virus (HIV) can infect nondividing cells, and integration of proviral DNA occurs without the need for cell division (30, 31). Indeed, HIV-based vector systems exploiting this property have recently been shown to transfer genes to nondividing cells (e.g., neurons) (39, 43), establishing "proof of principle."

Several features inherent to HIV create challenges for its development as a gene delivery vector. The fact that it is a known pathogen requires that the vector systems be completely free from even traces of replication-competent virus. Additionally, the complexity of the HIV genome and the key roles played by the viral regulatory (11) and accessory proteins (55) in modulating gene expression and viral infectivity make the development of efficient HIV-based vector systems a non-obvious venture.

A key HIV regulatory protein that has to be considered in the development of an HIV vector system is Rev. Rev is normally required to mediate nuclear-cytoplasmic transport of mRNAs encoding the HIV structural proteins (12, 15, 20, 33). In most circumstances, Rev would thus be expected to be needed in the packaging cell to allow the production of the

structural proteins as well as for production of full-length RNA from an HIV vector.

We have previously described the use of a small fragment from the Mason-Pfizer monkey virus (MPMV) genome, called the constitutive transport element (CTE), that allowed HIV structural protein synthesis without the need for Rev (6). This element also enabled the production of infectious HIV when introduced into a proviral clone lacking a functional *rev* gene. It should thus be possible to utilize this element to create an HIV vector system that does not require Rev. Such a system would be simpler to manipulate, and the Rev-independent HIV vectors could be also be used to deliver Rev antagonists such as Rev M10 (32, 38) for the inhibition of HIV infection.

The other regulatory genes, *tat* and *nef*, might also be expected to affect the titer of HIV vectors produced from packaging cells. *tat* would be expected to be necessary for the transcriptional activation of the viral long terminal repeat (LTR) (11) and possibly also for efficient reverse transcription in the target cell (21a). *nef* might be expected to increase the infectivity of vector produced from the packaging cell, in analogy to its effect on infectious virus (2, 41, 48, 49). In addition, the other HIV accessory genes (*vif*, *vpr*, and *vpu*) (55) might also have effects on gene transfer efficiency.

In this paper, we describe the generation of stable HIV type 1 (HIV-1)-packaging cell lines that constitutively express the HIV-1 structural proteins. Cell lines which produce structural proteins that efficiently package vector RNA either with or without Rev expression are described. These lines were used to generate HIV-1 vector stocks capable of delivering a selectable marker to CD4-positive target cells. The packaging cell system was also used to assess the effect of differential expression of *rev*, *tat*, and *nef* on the vector titer.

### MATERIALS AND METHODS

**Plasmid constructs.** Each plasmid described below is referred to by both name and number in the form of pHRxxxx to facilitate identification.

The plasmid pCMV (pHR16) has been previously described (29). Except as noted, all of its derivatives described below have HIV sequences positioned

\* Corresponding author. Phone: (804) 982-1599. Fax: (804) 982-1590. E-mail: dr4u@virginia.edu.

† Present address: Bone Marrow Transplant Division, Department of Medicine, University of Wisconsin, Madison, WI 53792.

immediately downstream of the promoter-enhancer from the simian cytomegalovirus (CMV) IE94 gene (bp 681 to 1349) (SCU38308 [GenBank Accession number M16019]) (24) and upstream of a spliceable intron and polyadenylation signal derived from the rabbit beta-globin gene (bp 906 to 1827) (RABHBB1A1 [GenBank accession number J00659]).

pCMVrev (pHR30) was described previously as pRev1 (29). It contains a *Bsu*36I fragment spanning the *rev* coding region from the HIV-1 cDNA clone pCV1 (bp 957 to 1678) (HIVPCV12 [GenBank accession number M11840]) (3) inserted into the plasmid pCMV (pHR16) as described above. pCMVtat (pHR136) is similar to pCMVrev except that it contains a *Sall*-*Bam*HI fragment, spanning the *tat* coding region from pCV1 (bp 787 to 1142), in place of the *rev* sequences. pCMVnef (pHR1405) was derived from pCMVrev by cloning *nef* sequences from pNL4-3 (bp 8787 to 9410) (HIVNL43 [GenBank accession number M19921]) (1) in place of *rev* sequences between the ATG at the start of *rev* and the *Bam*HI site present near the 3' end of the *rev* gene.

pCMVgagpol-RRE (pHR354) contains HIV sequences derived from the BH10 clone (19, 42) (bp 229 to 5332 followed by bp 7168 to 8021), and pCMVenv contains HIV sequences derived from the HxB2 clone (42) (bp 5550 to 8443). In both cases, the numbering system is given in terms of the HxB2 genome (HIVXB2R [GenBank accession numbers K03455 and M38432]). These plasmids are similar to the previously described pGAGPOL-RRE-r (pHR146) (51) and pSVSX1Δ1 (pHR278) (20) except that they contain the simian CMV immediate-early promoter in place of the simian virus (SV40) sequences.

pCMVgagpol-CTE (pHR1361) is similar to pCMVgagpol-RRE except that the Rev response element (RRE) sequences (HIV bp 7620 to 8474) and beta-globin intron have been replaced by the MPMV CTE and polyadenylation signal (bp 8007 to 8557) (SIVMPG [GenBank accession number M12349]) (6). pCMVenv-CTE (pHR1374) was created from pCMVenv by insertion of the MPMV CTE and polyadenylation signal (bp 8007 to 8540) in place of the beta-globin intron.

The HIV vector pTR167 (pHR1266) has been previously described (45) and was a gift from Nito Panganiban, University of Wisconsin, Madison. To make this vector, pNL4-3 (HIVNL43 [GenBank accession number M19921]) (1) was cleaved to completion with *Nsi*I and religated. This deleted the central portion of the HIV genome between nucleotides 1251 and 6742. A cassette containing the hygromycin B resistance gene under the control of the SV40 early promoter was then inserted into the *Nhe*I site near the middle of the *env* gene at nucleotide 7250. pTR167-ΔXho (pHR1572) was derived from pTR167 by cleavage and T4 DNA polymerase repair of its unique *Xba*I site corresponding to nucleotide 8887 of pNL4-3. This inactivated *nef* by the introduction of a frameshift mutation near the start of the open reading frame. pTR167-CTE contains the MPMV CTE (SIVMPMV) (bp 8007 to 8240) inserted into the repaired *Xba*I site as well as a small deletion of pNL4-3 nucleotides (8790 to 8886) immediately upstream of the CTE. These modifications also inactivated *nef*.

pHyg (pHR392), a plasmid which confers hygromycin B resistance (54), and pRSVneo (pHR1265) (SYNPRSVNEO [GenBank accession number M77786]), a plasmid which confers G418 resistance (17), have been previously described. All plasmids and further details about their construction are available on request.

**Maintenance of cell lines.** Cell lines were maintained in Iscove's medium (GIBCO) containing 10% calf serum (Hyclone). CMT3-COS cells are a line of African green monkey kidney cells expressing SV40 large T antigen under the control of the mouse metallothionein gene promoter (16). HeLa-CD4 cells (8) were kindly provided by D. Camerini, University of Virginia, Charlottesville, and were maintained in Iscove's medium containing G418 (1.5 mg/ml).

**Creation of cell lines stably expressing HIV structural proteins.** Cells were stably transfected by using a modification of the calcium phosphate method (18). In preparation for the transfection, cells were first plated onto 100-mm-diameter plates at a density of about  $2 \times 10^6$  cells per plate and incubated overnight. The transfection was performed the next day, when the plates were 70 to 80% confluent. At this time, the cells were washed twice with 10 ml of Tris-buffered saline (TS) (pH 7.4) (137 mM NaCl, 20 mM KCl, 25 mM Trizma base, 0.7 mM Na<sub>2</sub>HPO<sub>4</sub> [anhydrous], 0.9 mM CaCl<sub>2</sub> [anhydrous], and 0.5 mM MgCl<sub>2</sub> [6-hydrate]) prior to the addition of the DNA.

A 1.5-ml suspension containing the DNA was added per 100-mm-diameter plate. The DNA suspension was prepared from two separate solutions. For each 1.5 ml of suspension, the first solution (solution A) contained 0.15 ml of 1.25 M CaCl<sub>2</sub>, 0.15 ml of DNA mixture in H<sub>2</sub>O, and 0.45 ml of H<sub>2</sub>O. The second solution (solution B) contained 0.6 ml of H<sub>2</sub>O and 0.15 ml of 10× HEPES buffer, pH 7.25 (1.37 M NaCl, 5 mM KCl, 7 mM Na<sub>2</sub>HPO<sub>4</sub> [anhydrous], 10 g of glucose per liter, and 210 mM HEPES). Solution A was added slowly to solution B under continuous bubbling to form a fine precipitate. To create B4.14 cells, 8 μg of pCMVgagpol-RRE, 2 μg of pCMVrev, and 2 μg of pHyg were added per 100-mm-diameter plate of CMT3-COS cells. To create 5BD.1 cells, 8 μg of pCMVenv and 2 μg of pRSVneo were added per 100-mm-diameter plate of B4.14 cells. To create 2A.22 cells, 8 μg of pCMVgagpol-CTE, 8 μg of pCMVenv-CTE, and 2 μg of pRSVneo were added per 100-mm-diameter plate of CMT3-COS cells.

The 1.5 ml of DNA suspension was left on the cells for 2 min, and then 1.5 ml of complete medium was added. After an additional 15 min of incubation at room temperature, 6 ml more of medium was added, and the cells were then incubated for an additional 5 h at 37°C in a 5% CO<sub>2</sub> incubator. The precipitate and medium were then removed, and 4 ml of 20% glycerol in TS was added. The glycerol solution was left on the cells for precisely 70 s. The cells were then

washed twice with 10 ml of TS before 10 ml of complete medium was added. The cells were then incubated at 37°C in 5% CO<sub>2</sub> overnight.

The plate of transfected cells was then split into six plates which were incubated in complete medium until the next day, when the appropriate drug selection was started. During the selection period, medium containing the drug was changed every 3 to 4 days. G418 was used at 1.5 mg/ml. Hygromycin B was used at 200 μg/ml. In either case, small resistant colonies were observed starting at about 8 to 10 days after the start of selection. The cell colonies were picked into 24-well plates at about day 14, expanded further as necessary, and screened for *gag* expression by p24 enzyme-linked immunosorbent assay (ELISA) or *env* expression by Western blotting. Each transfection yielded numerous positive clones.

**Transient transfection of packaging cells and infection of target cells.** Packaging cells were transfected to produce a vector stock, using a DEAE-dextran transfection method previously described (21). Typically, a 100-mm-diameter plate of cells was transfected with 5 μg of HIV vector (pTR167 and its derivatives) and 2 μg of each of the pCMV-based vectors expressing the various HIV regulatory proteins. When pCMVenv was cotransfected, 20 μg was used. Supernatants were harvested at 72 h posttransfection and cleared of cells by centrifugation at 2,500 rpm in an IEC Centra-8R centrifuge at 4°C for 15 min.

To perform the infection of HeLa-CD4 cells with the HIV vector stocks, 10-fold serial dilutions of the cleared supernatant were made in complete medium. DEAE-dextran was then added to each dilution at a concentration of 8 μg/ml to facilitate viral absorption, and 1 ml of each dilution was added to a 60% confluent 60-mm-diameter dish of cells, which had been subcultured the previous day. The vector was allowed to adsorb for 4 h at 37°C, at which time 4 ml of complete medium was added and the incubation was continued. The medium was replaced 2 days later with medium containing 200 μg of hygromycin B per ml. This medium was changed every 2 to 3 days. After about 14 days, the resultant colonies were fixed and stained with 0.5% crystal violet in 50% methanol. All experiments were carried out in duplicate.

To test for replication-competent virus that might be produced from the packaging cells, medium was collected from the transfected cell cultures and 6 ml of the collected medium was used to infect  $5 \times 10^6$  MT-4 cells. Virus was allowed to adsorb for 24 h in the presence of 8 μg of DEAE-dextran per ml. The cells were then spun down, washed one time in phosphate-buffered saline, and resuspended in 10 ml of RPMI plus 10% fetal calf serum. Thereafter, at 3- to 4-day intervals, two-fifths of the culture (4 ml) was collected for p24 assay and replaced with fresh medium.

**Western blots and p24 assays.** Western blotting was performed as previously described (20, 21) either with a monoclonal antibody directed against p24 (9), Rev (40), or Vif (50) or with a rabbit polyclonal antiserum directed against gp120. The rabbit serum was produced by immunization with a fragment of gp120 (amino acids 343 to 512) produced in *Escherichia coli*. p24 assays were performed with a commercial kit (DuPont) according to the directions of the manufacturer.

## RESULTS

**Creation of packaging cell lines that constitutively express the HIV-1 structural protein genes.** The first step in the generation of a helper-free vector system is the establishment of cell lines that can be used to package vector-derived RNA. To create such cell lines for use with HIV vectors, one must take into account the fact that expression of the HIV-1 structural protein genes normally requires the presence of the RRE in *cis* and coexpression of the HIV-1 *rev* gene in *trans*.

We have previously described transient-expression systems containing these components which allowed production of large quantities of HIV-1 pseudovirions (51) and envelope proteins (44). Using these systems, we demonstrated that virus-like particles could be produced from transfected CMT3-COS cells and that cell surface envelope protein expression could be readily obtained. Subsequently, we showed that expression of structural protein genes from these vectors could be made Rev independent by insertion of the MPMV CTE into the respective expression vectors (6). This element consists of a 168-nucleotide fragment derived from the 3' end of the MPMV genome (13, 14). The CTE overcomes the need for Rev coexpression, presumably by interacting directly with a cellular protein involved in RNA export.

Our original plasmid expression systems were based on SV40 late replacement vectors that contained the entire SV40 early region and required SV40 replication for HIV-1 protein expression. Because SV40 replication kills transfected cells,

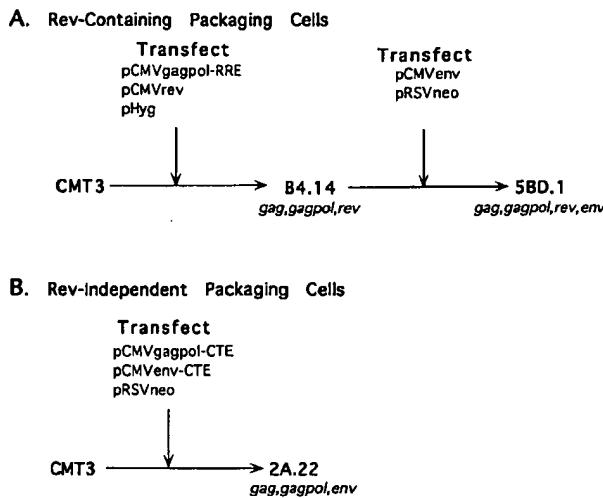


FIG. 1. Scheme for the production of stable packaging cells. (A) Rev-containing packaging cells were produced from the parental CMT3 cell line in two steps by transfection with the indicated plasmids. These cells are hygromycin B and G418 resistant. (B) Rev-independent packaging cells were produced in a single step by transfection with the indicated plasmids. These cells are G418 resistant. The HIV genes contained in each cell line are given in italics under the cell line name.

these plasmids were not suitable for the creation of stable cell lines. To create expression plasmids that could be used for this purpose, we modified our original vectors by exchanging the SV40 sequences for the simian CMV immediate-early promoter. This led to the creation of four novel plasmids: CMVgagpol-RRE and pCMVenv, which require Rev coexpression for HIV-1 structural gene expression, and pCMVgagpol-CTE and pCMVenv-CTE, which do not. (see Materials and Methods for plasmid details). These plasmids were structurally similar to pCMVrev, a plasmid which expresses HIV-1 Rev, which has been previously described (29). Similar vectors that expressed Tat (pCMVtat) and Nef (pCMVnef) were also made.

RNA produced from these plasmids would not be expected to be packaged into viral particles, since sequences from the 5' end of the genome, which are required for packaging, are lacking in every case. The constructs expressing *env*, *nef*, *tat*, and *rev* are all devoid of any of the sequences that have been implicated in packaging. The plasmids expressing *gag-pol* do contain some of the region thought to be required for packaging but lack the first 228 nucleotides of the HIV genome. This includes all of R, all of U5, and the primer binding site. Several studies have shown that these sequences form part of the RNA packaging signal (5, 34, 56), and HIV RNA lacking these sequences packages at least 15 times less well than the wild type (22). The expression plasmids also lack some of the sequences required for reverse transcription.

To create Rev-containing and Rev-independent packaging cell lines, CMT3 cells were transfected with vectors expressing Gag, Gag-Pol, and Env, using a calcium phosphate transfection procedure. The lineages of these cell lines are shown in Fig. 1. Rev-containing B4.14 cells were made by cotransfection of pCMVgagpol-RRE, pCMVrev, and pHyg, a plasmid that confers resistance to hygromycin. This cell line was then used as transfection recipient to create the 5BD.1 cell line. To make 5BD.1 cells, B4.14 cells were cotransfected with pCMVenv and pRSVneo, a plasmid that confers resistance to G418. 5BD.1 cells are thus resistant to both hygromycin and G418. The

Rev-independent cell line 2A.22 was made by cotransfection of pCMVgagpol-CTE, pCMVenv-CTE, and pRSVneo.

All three cell lines were initially assessed for HIV-1 structural protein gene expression. HIV-1 envelope protein expression was determined by analysis of cell lysates on Western blots, using a gp120-specific antiserum (Fig. 2A). As expected, both 5BD.1 and 2A.22 cells expressed high levels of gp160 and gp120. A similar analysis of the cell lines with an anti-Rev antiserum demonstrated that B4.14 and 5BD.1 cells also expressed Rev (data not shown).

A quantitative p24 (CA) assay was performed to analyze the levels of HIV-1 Gag expression in the cell lines. To do this, a confluent plate of each cell line was split 1:5 and allowed to grow at 37°C for 3 days. Samples of media were taken at 1-day intervals during this time period and assayed for p24 by ELISA. The results of this assay are shown in Fig. 2B. All three cell lines excreted large amounts of p24, reaching levels of between 10 and 30 ng/ml after 3 days in culture. The 5BD.1 cell line consistently showed threefold-higher levels than the other two. In addition, Western blot analyses with either a human

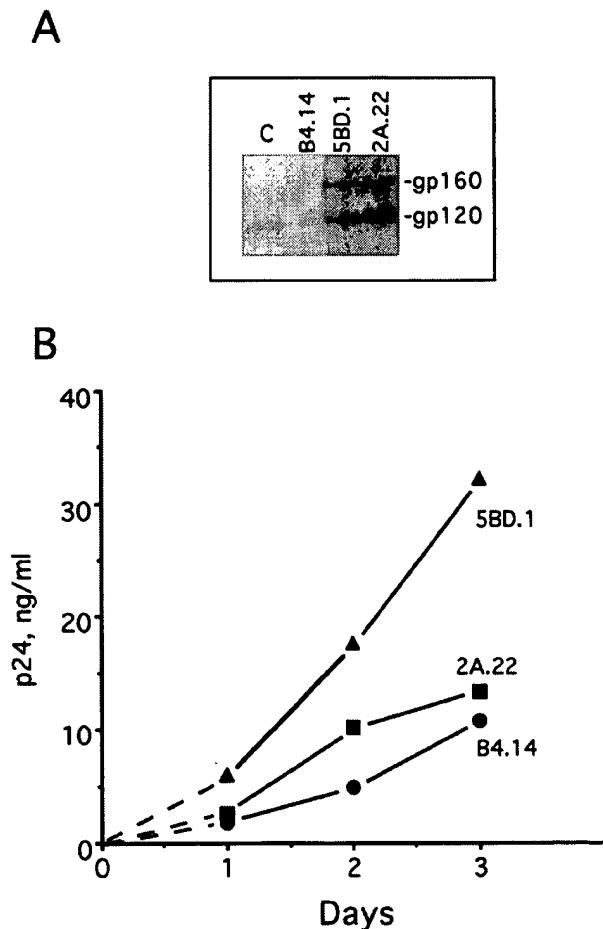


FIG. 2. Analysis of *env* and *gag-pol* expression in the stable packaging cell lines. (A) Western blot of lysates from about 10<sup>6</sup> cells of each indicated cell line. The primary antibody consisted of a 1:300 dilution of polyclonal rabbit antiserum directed against gp120. A goat anti-rabbit secondary antibody, conjugated to alkaline phosphatase, was used to develop the blot. C, CMT3 cell line control. (B) p24 production by the stable cell lines as a function of days in culture. Supernatants from each cell line were analyzed for p24 antigen by using a DuPont ELISA test kit as described in the text.

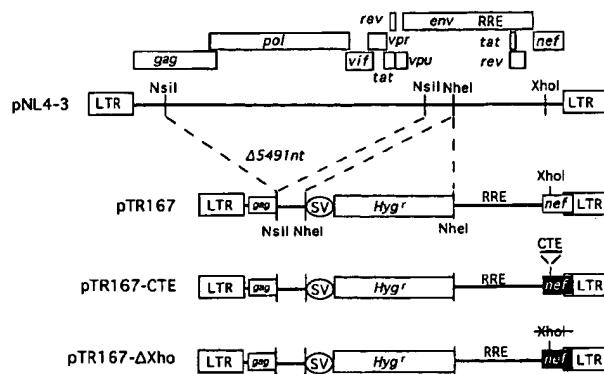


FIG. 3. HIV vectors used to transfer hygromycin B resistance. The top portion is a representation of the pNL4-3 genome showing the open reading frames, 5' and 3' LTRs, and restriction enzyme sites relevant to the construction of the vectors. The proviral portion of pTR167 and its derivatives are shown below this diagram. The inactive *nef* gene of pTR167-CTE and pTR167-ΔXho is shown as a filled box (see text for details).

HIV-positive antiserum or a monoclonal antibody directed against p24 (CA) showed good expression of the Gag and Gag-Pol gene products within all three cell lines (data not shown).

The Gag-Pol expression plasmids used to make cell lines also contained the Vif open reading frame and were thus theoretically capable of expressing Vif from a spliced mRNA. To assay for Vif expression, a Western blot analysis was performed with each cell line by using an anti-Vif monoclonal antibody obtained from Michael Malim (University of Pennsylvania). Low-level Vif expression could be detected in the 5BD.1 cell line, but Vif expression was not detectable in either the B4.14 or 2A.22 cell line (data not shown). This result was surprising because 5BD.1 is derived from B4.14. However, since our p24 analysis demonstrated that 5BD.1 cells express higher levels of Gag-Pol than B4.14 cells, Vif may be expressed in B4.14 at levels below detection by the method used.

**The B4.14, 5BD.1, and 2A.22 cell lines can be used to package HIV-1 vector-derived RNA without production of replication-competent virus.** The cell lines were next tested for their abilities to package viral RNA derived from an HIV vector and promote the production of virus particles capable of delivering a gene to target cells. To do this, we utilized a previously described HIV vector, pTR167 (45). pTR167 is derived from the infectious HIV-1 proviral clone pNL4-3 (1), and a schematic diagram of the important portion of this vector is shown in Fig. 3. pTR167 was created by deletion of 5,491 nucleotides from the central region of pNL4-3, followed by the insertion of an SV40 early promoter-driven hygromycin resistance cassette in the middle of the *env* region. The vector retains both LTRs, the *nef* gene, the 3' end of the *env* gene including the RRE, and sequences from the 5' end of the genome just into the start of p24.

Transfection of packaging cells with pTR167, together with vectors that produce Tat and Rev (e.g., pCMVrev and pCMVtat), would be expected to produce three pTR167-derived RNA species. The largest of these would be a full-length RNA produced from the viral LTR. This RNA should be capable of being packaged into HIV particles and would also be expected to be spliced into a subgenomic species, since the major 5' splice donor and 3' *tat*-*rev* splice acceptor sites present in the HIV genome remain intact. The spliced mRNA should encode Nef. Indeed, Western blot analysis of cells transiently

transfected with pTR167, pCMVrev, and pCMVtat confirmed that Nef was abundantly produced (data not shown). The third RNA species would be expected to be an mRNA encoding hygromycin resistance produced from the internal SV40 early promoter. Infection of target cells with HIV particles containing full-length pTR167 RNA would be expected to lead to integration of a vector-derived provirus and expression of hygromycin resistance, assuming that expression of the SV40 promoter-driven mRNA was Tat and Rev independent.

To test the packaging cell-vector system, pTR167 and pCMVtat were transfected into 2A.22, B4.14, or 5BD.1 cells, using DEAE-dextran. In some cases pCMVenv was also cotransfected. pCMVrev was also added to the transfections involving the 2A.22 cells, since this cell line did not contain endogenous Rev. Medium from each transfected plate was harvested at 72 h posttransfection. One milliliter of the collected medium was then used to infect HeLa or HeLa-CD4 cells, according to the procedures described in Materials and Methods. The infected cells were then subjected to hygromycin selection for 14 days, at which time they were stained and colonies were counted. The results of this experiment are presented in Table 1.

The data in Table 1 demonstrate that both the 2A.22 and 5BD.1 cell lines supported the production of infectious vector which could deliver the hygromycin resistance gene to the HeLa-CD4 target cells with titers greater than 10<sup>3</sup> CFU per ml. Gene delivery was clearly HIV envelope protein mediated, since infection of HeLa cells lacking CD4 did not occur. The B4.14 cell line also produced vector capable of delivering the marker gene to HeLa-CD4 cells at reasonably high titers, but only when a source of HIV-1 envelope protein was provided by cotransfection with pCMVenv. pCMVenv cotransfection had little effect on vector production from the 2A.22 cell line.

We next performed an experiment designed to determine if replication-competent virus was produced in the packaging cell lines transfected with pTR167. It seemed unlikely that this would occur, since the plasmids used to create the packaging cells lacked viral LTRs and had no regions of overlap (except for the RRE or CTE). Multiple recombination events between the inserted genes and pTR167 would thus have to take place to create a replication-competent virus (see Discussion). Nevertheless, because of the safety issues involved, we wanted to formally rule out this possibility.

To test for replication-competent virus production, a 100-mm-diameter plate of each of the three cell lines was cotransfected with pTR167, pCMVrev, pCMVtat, and pCMVnef. pCMVenv-CTE was also added to the cotransfection for the B4.14 cell line. After 72 h, the medium (10 ml) from each plate was collected. In each case we also collected medium from a plate that was not subjected to the transfection procedure. Six

TABLE 1. Titers of pTR167 packaged in different cell lines with or without added HIV envelope protein

Cell line	pCMVenv	Titer (CFU/ml) <sup>a</sup> on:	
		HeLa cells	HeLa-CD4 cells
2A.22 <sup>b</sup>	-	0	2,480 ± 50
	+	0	1,840 ± 110
B4.14	-	0	0
	+	0	800 ± 200
5BD.1	-	ND <sup>c</sup>	9,000 ± 1,800

<sup>a</sup> Results are means ± standard deviations for duplicate determinations.

<sup>b</sup> Transfections also contained pCMVrev.

<sup>c</sup> ND, not determined.

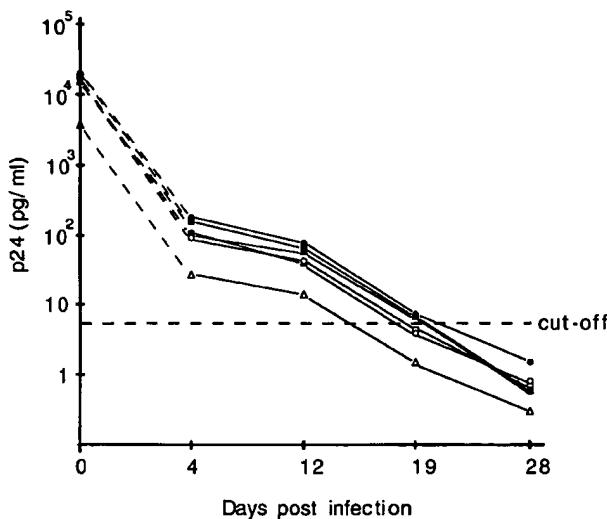


FIG. 4. p24 antigen assay to detect replication-competent virus. MT-4 cells were infected with supernatants from various cell lines that either were transfected with pTR167 and the additional expression vectors described in the text (closed symbols) or were not transfected (open symbols). p24 was assayed throughout the experiment at the days indicated. Supernatants were from B4.14 (circles), 2A.22 (triangles), or 5BD.1 (squares) cells. The dashed line represents the calculated sensitivity cutoff as specified by the ELISA kit's manufacturer.

milliliters of the collected medium from each plate was then used to infect  $5 \times 10^6$  MT4 cells as described in Materials and Methods. The MT4 cell line is a line of human T-cell leukemia virus type 1-transformed CD4-positive T cells that are extremely permissive for growth of HIV isolates containing an HIV-1<sub>IIIB</sub> envelope protein (data not shown). The infected cells were passaged for 28 days under conditions that would support the growth of virus. At regular intervals, medium was collected and assayed for p24 by ELISA. The results of this experiment are shown in Fig. 4.

Analysis of Fig. 4 reveals that all six infected cultures behaved essentially the same. There was no significant difference between the infections derived from the cell lines that were cotransfected with the collection of plasmids and those that were not. In all cases, input p24 was reduced by about 100-fold at 4 days postinfection (the initial infection procedures washed away some of the input p24). The decline continued to below detectable levels by about 19 days. In no case could replication-competent virus be detected.

**The presence of Tat in the producer cell enhances vector titer.** Expression of the full-length RNA in pTR167 is directed by the viral LTR. It was therefore of interest to determine if the presence of Tat in the producer cell influenced vector titers, since Tat would be expected to transactivate the viral LTR. Tat has recently also been shown to be required for efficient reverse transcription in newly infected cells (21a).

To test for the consequence of Tat expression on the vector titer, 5BD.1 and 2A.22 cells were transfected with pTR167 with or without pCMVtat cotransfection. For this experiment, pCMVrev and pCMVnef were also added to all of the transfections. This controlled for any possible effect that Rev or Nef may have on the vector titer and allowed us to examine only the effect of Tat. Medium was harvested at 72 h posttransfection and used to infect HeLa-CD4 cells, which were subjected to hygromycin selection for 14 days. Colonies were then counted, and the results are tabulated in Table 2. When pCMVtat was present in the producer cells, titers of about 5  $\times$

TABLE 2. Effect of Tat expression on vector titers in Rev-containing and Rev-independent packaging cells

Cells and vector	pCMVrev	pCMVtat	pCMVnef	Titer (CFU/ml) <sup>a</sup>
5BD.1 cells, pTR167	+	+	+	4,500 $\pm$ 100
	+	-	+	650 $\pm$ 250
2A.22 cells, pTR167	+	+	+	5,250 $\pm$ 450
	+	-	+	800 <sup>b</sup>

<sup>a</sup> Results are means  $\pm$  standard deviations for duplicate determinations.

<sup>b</sup> Single determination.

10<sup>3</sup> CFU/ml were achieved with both cell lines. In the absence of Tat, the vector titers were approximately sevenfold lower.

**Rev is required in the producer cell in trans for good vector titers but can be replaced by providing the CTE in the vector in cis.** To test whether good vector titers were dependent on the presence of Rev in the producer cell, a transfection-infection experiment similar to the one described above was performed. In this instance, pTR167 was cotransfected into the packaging cell line 5BD.1 or 2A.22 with or without pCMVrev. For this experiment, pCMVtat and pCMVnef were also added to all of the transfections. This controlled for any possible effect that Tat or Nef may have on vector titer and allowed us to examine only the effect of Rev. Results from the subsequent infection of the HeLa-CD4 cells with the producer cell supernatants are shown in Table 3.

The deletion of pCMVrev from the transfection of 5BD.1 cells did not have any effect on the titer of the vector produced from these cells. However, in the case of 2A.22 cells, deletion of pCMVrev reduced the vector titer to near zero. These results suggested that the vector required Rev but that 5BD.1 cells contained enough endogenous Rev to obtain good titers.

To further establish this, we created a modified version of pTR167, pTR167-CTE, which had the MPMV CTE cloned into the *Xba*I site of Nef. The relevant portion of this plasmid is shown in Fig. 3. When this vector was tested in the transfection-infection protocol, good titers were obtained in both 5BD.1 and 2A.22 cells, with or without added Rev. Thus, we can conclude that expression from pTR167 is Rev dependent but that the need for Rev coexpression can be completely overcome by insertion of the CTE into the vector. It is interesting that the titers obtained from the system completely lacking Rev (2A.22/pTR167-CTE) are roughly equivalent to the titers obtained with Rev.

TABLE 3. Effect of Rev expression on vector titers in Rev-containing and Rev-independent packaging cells

Cells and vector	pCMVrev	pCMVtat	pCMVnef	Titer (CFU/ml) <sup>a</sup>
5BD.1 cells	+	+	+	4,500 $\pm$ 100
	-	+	+	5,600 $\pm$ 900
	+	+	+	3,300 $\pm$ 200
	-	+	+	2,950 $\pm$ 550
2A.22 cells	+	+	+	5,250 $\pm$ 450
	-	+	+	1.5 $\pm$ 0.5
	+	+	+	3,950 $\pm$ 650
	-	+	+	3,400 $\pm$ 700

<sup>a</sup> Results are means  $\pm$  standard deviations for duplicate determinations.

TABLE 4. Effect of Nef expression on vector titers in Rev-containing and Rev-independent packaging cells

Cells and vector	pCMVrev	pCMVtat	pCMVnef	Titer (CFU/ml) <sup>a</sup>
<b>5BD.1 cells</b>				
pTR167	+	+	+	4,500 ± 100
	+	+	-	3,400 ± 300
pTR167-CTE	+	+	+	3,300 ± 200
	+	+	-	650 ± 350
pTR176-ΔXho	+	+	+	1,850 ± 550
	+	+	-	700 ± 400
<b>2A.22 cells</b>				
pTR167	+	+	+	5,250 ± 450
	+	+	-	3,100 <sup>b</sup>
pTR167-CTE	+	+	+	3,950 ± 650
	+	+	-	505 ± 25
pTR167-ΔXho	+	+	+	7,700 ± 1,200
	+	+	-	550 ± 150

<sup>a</sup> Results are means ± standard deviations for duplicate determinations.

<sup>b</sup> Single determination.

**The presence of Nef in the producer cell enhances vector titer.** As mentioned above, pTR167 contains an intact *nef* gene and expresses a functional Nef protein. To determine the effect of Nef expression on vector titer, we used two *nef*-negative derivatives of pTR167. The first of these, pTR167-ΔXho, was made by cleavage of pTR167 with *Xba*I followed by subsequent repair with T4 DNA polymerase. This causes a frameshift to occur near the start of the *nef* open reading frame. The relevant portion of this plasmid is shown in Fig. 3. We also used pTR167-CTE in these experiments, since it contains a nonfunctional *nef* gene due to the insertion of the CTE into the *nef* open reading frame.

To examine the consequence of Nef expression in the producer cell on the subsequent vector titer, 5BD.1 or 2A.22 cells were transfected with either pTR167, pTR167-CTE, or pTR167-ΔXho, with or without pCMVnef cotransfection. For this experiment, pCMVrev and pCMVtat were also added to all of the transfections. This controlled for the effects that Rev or Tat have on the vector titer and allowed us to examine only the effect of Nef. The results of this experiment are shown in Table 4.

As expected, pCMVnef had little effect on the vector titer in the case of pTR167. However, for pTR167-CTE as well as pTR167-ΔXho, pCMVnef cotransfection increased vector titers significantly (3- to 14-fold). This was true in both the Rev-dependent and Rev-independent cell lines.

## DISCUSSION

We have described cell lines that constitutively express HIV-1 structural proteins and have demonstrated that they can be used to package HIV vector RNA. These lines were readily created by standard transfection protocols. In contrast to the ease with which we made these lines, others have shown that constitutive expression of the HIV-1 envelope protein (52) and protease (25, 27) is toxic and have concluded that stable lines expressing these proteins are not easily obtainable (27). A recent report described the creation of an inducible packaging cell line which was designed to overcome this perceived problem (58).

It is hard to reconcile our ability to easily create cell lines constitutively expressing the HIV structural proteins with the difficulties encountered by others. There does not appear to be

anything unique about the parental cell line used to establish these packaging cells that would explain this. Also, we have recently created similar packaging lines from a different parental line (293-T) by the same method (data not shown).

One possible reason for our success in creating packaging lines may be that the selectable marker was introduced on a plasmid separate from the ones expressing the structural protein genes. It is possible that this more readily allows for the selection of cells producing tolerable levels of the structural proteins, since it enables covariation. Consistent with this notion, another cell line that constitutively expresses HIV structural proteins has recently been described (10). To create this, a full-length proviral clone lacking only 37 nucleotides from the packaging region was cotransfected with a plasmid expressing a *neo* marker. This line was also shown to package a minimal HIV vector with an efficiency similar to that of our line.

Several properties of our packaging cells probably account for the fact that we were unable to detect replication-competent virus. First, the cell lines were constructed by using a split genome approach, with the *gag-pol-vif* coding region on one plasmid and the *env* region on another. There is also virtually no overlap between the two plasmids except for the RRE or CTE. In the 2A.22 and 5BD.1 cell lines, one or the other of these sequences is present on both constructs, but the sequences are positioned such that a recombination event within them would not generate a complete genome. Both plasmids also lack almost all of the *cis*-acting signals needed for viral replication, including the viral LTRs. In addition, with the exception of *vif*, the viral regulatory and accessory genes are made from additional separate plasmids. Thus, multiple recombination events would have to occur between the stably transfected DNA and vector DNAs to produce an infectious recombinant.

The vector titer obtained for HIV-1 Env-mediated gene transfer to CD4-positive cells is comparable to titers recently published by others ( $10^3$  to  $10^4$  CFU/ml) (10, 58). We obtained this titer without using any of the titer enhancement protocols that have been described in the literature, such as harvest at 32°C (26), spin infection (26), or pseudotyping with vesicular stomatitis virus envelope glycoprotein (VSV G) followed by concentration by ultrafiltration (43). It seems likely that incorporation of some of these methodologies into our protocols will lead to higher vector titers. In this regard, preliminary experiments using the B4.14 cell line have demonstrated that it can be used to create HIV pseudotypes that could be concentrated and used to expand the host range of our vectors.

Our experiments also assessed the role of Tat, Nef, and Rev in vector titer. The presence of Tat and Nef in the producer cell each increased vector titer about 5- to 10-fold. Rev, on the other hand, was absolutely essential for any titer, unless the MPMV CTE was included in the vector.

The increase in titer observed in the presence of Tat could be due to transactivation of the viral LTR present in the vector or to the recently described effect of Tat on reverse transcription (21a), or both. Activation of transcription from the viral LTR would be expected to lead to increased amounts of full-length RNA for packaging. The rather low magnitude of the effect (5- to 10-fold) compared to that in other reports describing Tat transactivation (23, 37) may be due to the fact that our HIV vector (pTR167) contained an SV40 origin of replication and our packaging cell line contained SV40 T antigen. Under these conditions, we have found the HIV LTR to be only partially dependent on Tat (unpublished data). The enhancement by Tat is also considerably lower than its reported effect on reverse transcription (21a). It is possible that the low enhance-

ment is partially the result of the fact that Tat was supplied by cotransfection that was not 100% efficient. Expressing Tat as a stable gene in a cell line might be expected to increase the titer further.

On the other hand, Tat is not needed in the target cell for the expression of the selectable marker, since the internal SV40 early promoter present in our plasmid is not dependent on Tat. It may thus be possible to distinguish between an effect of Tat on transcription in the producer cell and an effect of Tat on reverse transcription in the target cell by designing a vector with a modified LTR. Modifications which alter the HIV LTR to make it constitutively active at high levels have been described (46). We are presently examining whether such modifications can lead to increased vector titers in the absence of Tat.

The mechanism by which Nef increased the vector titer is not known, although our observation of a 5- to 10-fold increase is consistent with reports in the literature that Nef increases viral infectivity (2, 41, 48, 49). Those reports suggest that Nef may play a role early in the virus life cycle, since Nef seems to enhance the reverse transcription process. However, it is at present unclear whether Nef exerts its effect in the producer cell to modify the viral structural proteins or whether Nef functions in the target cell. The latter possibility is supported by recent reports that have demonstrated small amounts of Nef in virus particles (7, 41, 57). The vector system described here is likely to provide a useful tool for examining this aspect of Nef function.

By creating an HIV vector which contained the MPMV CTE (pTR167-CTE) and a packaging cell line which expressed the HIV structural proteins in a Rev-independent fashion (2A.22), we were able to obtain an HIV vector system that functions completely without Rev. The titer of the vector obtained from this system was essentially the same as that obtained from a parallel system which contained Rev. In this context the CTE seemed to substitute completely for Rev-RRE function, similar to what was previously observed in transient-expression assays with Rev-dependent constructs (6). This is in contrast to situations where several rounds of HIV replication were measured. In those cases, titers from CTE-containing viruses were always reduced by at least 1 log unit compared to viruses utilizing Rev and the RRE (6, 59). We presently do not have an explanation for this difference.

Our ability to create an HIV vector system that works in the absence of Rev opens the possibility of using it as a delivery vehicle for intracellular immunization (4) against Rev function. This is not possible to do with a vector system that is dependent on Rev, since the antagonist would inhibit vector production. Several Rev antagonists that have dramatic inhibitory effects on HIV replication, such as Rev M10 (32, 38) or RRE decoys (28), have been described. These genes could be readily introduced into an HIV vector and put into cells normally infectable by HIV. Expression of the "anti-Rev" gene would be expected to dampen HIV infection. Any residual HIV replication should lead to activation of the vector LTR (by Tat) and create a vector-derived RNA that would be packaged by proteins derived from the infectious virus. In this scenario, the wild-type virus would act as a helper that may allow the spread of vector particles to previously nonimmunized cells. Because of the additional vector spread, it is likely that this type of scheme will be more effective in modulating HIV infection *in vivo* than one based on traditional retrovirus vectors. We are currently testing this approach in model systems.

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